University of Leicester

The Nociceptin System in Inflammation and Sepsis

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Nociceptin/OrphaninFQ, N/OFQ, and its receptor NOP represent a non-opioid branch of the opioid family. There is growing interest in the involvement of this system during inflammation and sepsis as it is present in immune cells and modifies immunocyte function. Systemic N/OFQ increased mortality in an animal model of sepsis and there is limited evidence for increased plasma N/OFQ in patients with sepsis who died compared to those who survived. This thesis explores changes in the expression of NOP and ppN/OFQ-mRNA by polymorphs (PMN) and of N/OFQ peptide in plasma during inflammation and sepsis. A further aim was to investigate the relationship between the N/OFQ system with physiological and biochemical indicators of severity of disease.

Forty patients undergoing cardiopulmonary bypass (CPB) and 49-patients with sepsis in the Intensive Care Unit (ICU) were recruited into 2-studies. In the CPB study we observed a 57% reduction of NOP-mRNA and a 95% reduction of ppN/OFQ-mRNA expression in PMN. Plasma N/OFQ concentrations increased by over 30%. Higher plasma N/OFQ was associated with lower NOP-mRNA. These changes were related to prolonged aortic cross clamp time. In patients with sepsis there was an 85% reduction of ppN/OFQ-mRNA expression compared to a sample taken after recovery from sepsis. Lower expression of ppN/OFQ-mRNA was associated with increased inotropic support and lactate concentrations on the first day of sepsis. Our data did not show any differences amongst survivors and non-survivors.

During inflammation(CPB) and sepsis there was reduced expression of NOP and ppN/OFQ-mRNA with an inverse relationship between plasma N/OFQ(CPB study) and NOP-mRNA expression, suggestive of a possible feedback mechanism. Based on the current evidence (this thesis and literature) we suggest that N/OFQ could contribute to the complex pathophysiological process occurring during inflammation and sepsis and warrant further study.

(300words)

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List of Abbreviations

ALT	Alanine transaminase
APACHE	Acute physiology and chronic health evaluation
AXC	Aortic cross clamp
bpm	Beats per minute
BSA	Bovine serum albumin
COPD	Chronic obstructive pulmonary disease
CPB	Cardio-pulmonary bypass
cpm	Counts per minute
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
Ct	Cycle threshold
CVVH	Continuous veno-venous hemofiltration
dl	Decilitre
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
FBS	Foetal Bovine Serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hours
Hb	Haemoglobin
HR	Heart rate

IABP	Intra aortic balloon pump
IFNγ	Interferon gamma
IHD	Ischaemic heart disease
IL-1Ra	Interleukin-1 receptor anatagonist
IPPV	Intermittent positive pressure ventilation
iu	International units
kPa	Kilopascals
MAP	Mean arterial pressure
MCP	Monocyte chemo-attractant protein
mg	Milligrams
ml	Millilitres
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NIPPV	Non-invasive positive pressure ventilation
N/OFQ	Nociceptin/Orphanin FQ peptide
NOP	Nociceptin/Orphanin FQ receptor
ORL_1	Nociceptin.Opioid Receptor-Like
PCT	Procalcitonin
PBS	Phosphate Buffered Saline
$pg.ml^{-1}$	picograms per ml
PI	Peptidase inhibitor
PMN	Polymorphonuclear leucocytes

ppN/OFQ	pre-pro N/OFQ
RR	Respiratory rate
RIA	Radioimmunoassay
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential Organ Failure Assessment
TGF	Transforming Growth Factor
TFA	Trifluoroacetic acid
TNFα	Tumour necrosis factor alpha
µl	Micro litres
VEGF	Vascular endothelial growth factor
WCC	White cell count

1 Introduction

1.1 The N/OFQ System

The nociceptin system comprise the peptide nociceptin/orphanin FQ (N/OFQ) and the N/OFQ receptor (NOP). N/OFQ is a 17 amino acid opioid-like peptide (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) first described in 1995[1;2] with structural similarity to the endogenous κ (or KOP)-opioid dynorphin A. N/OFQ is produced and released by neurons and other tissues from a 176 amino acid precursor pre-pro N/OFQ (ppN/OFQ).[3] Other peptides derive from the N/OFQ precursor ppN/OFQ, currently Orphanin FQ2 (biologically active), and Nocistatin (functional antagonist of N/OFQ) have been identified. Neither of these peptides bind the NOP receptor.[4]

The metabolism of N/OFQ (also known as Noc or NC) has been investigated in animal and in human *in vitro* studies. In mouse brain cortex, aminopeptidase N metabolises N/OFQ to Noc (2-17); endopeptidase is also involved in the metabolism of N/OFQ. Noc (1-13) and Noc (1-9) metabolites have been observed in rat hypoccampus;[4] these substances are inactive.

In humans, N/OFQ concentrations have been measured in plasma, CSF and synovial fluid in healthy controls and patients with a variety of conditions involving acute and chronic pain. Plasma concentrations of N/OFQ in healthy controls have been reported between 2.5 - 15 $pg.ml^{-1}$.[5-7] Yu and colleagues studied the metabolism of N/OFQ in human blood. They sampled incubated human blood with

N/OFQ at 15 minute intervals for to 2 hours, and observed multiple metabolites (mainly Noc (2-17) that had similar pattern to the metabolism of dynorphin A. It is unknown what the end products and half-life of N/OFQ *in vivo* is in humans, and whether they are active or not.

NOP was cloned in 1994 and is an inhibitory G protein coupled receptor (GPCR). It is a member of the opioid receptor family (International Union of Basic and Clinical Pharmacology, IUPHAR).[8] NOP is distributed widely in the central[9], peripheral and sensory nervous systems, as well as other peripheral tissues.[10;11] In the human brain, NOP is found all throughout, but more abundant in cortical areas, hypothalamus, thalamus, and striatum.[9] In the rat spinal cord it is located in the grey matter of dorsal and ventral horns.[12] In peripheral tissues NOP has been identified in human right atrium,[11] immune cells[9;13-17] mouse splenic lymphocytes, rat intestine, porcine gastrointestinal tract and kidney, and guinea-pig sympathetic ganglia.[12]

Much of the original work on the N/OFQ-NOP system centered around its complex involvement in pain pathways: N/OFQ produces analgesia when administered at spinal sites and hyperalgesia/anti-opioid actions when administered supraspinally.[18] N/OFQ also causes hypotension and bradycardia when administered at central and peripheral sites (central: intracerebroventricular, rostral ventrolateral medulla, and paraventricular nucleus of the hypothalamus. Peripheral: intravenous, and into mesenteric vessels).[19-21] The effect of N/OFQ on gastric motility and acid secretion by the stomach varies depending on whether it is

administered central or peripherally. Intracerebroventricular administration of N/OFQ inhibits contractility of the gastro-intestinal tract and reduces gastric acid secretion; on the other hand, intraperitoneal N/OFQ increases gastric acid secretion without changes on gastric emptying.[18] Various other functions have been attributed to N/OFQ as seen in **Figure 1-1**. It is unclear why some of the N/OFQ and other NOP agonists actions are different and even opposite when administered centrally vs peripherally. The extensive location of NOP throughout the body and the diversity of effects that NOP ligands can cause in multiple systems, will pose a challenge when the time comes to develop pharmacological agents for the treatment of specific conditions.

There is now good evidence to support a role for this relatively new peptide-receptor system in regulating other physiological functions, e.g. the cardiovascular, hypothalamic-pituitary-adrenal (HPA) axis and immune systems. Binding of N/OFQ to NOP causes a variety of cellular actions (**Figure 1-2**), which in neurones reduces excitability and transmitter release (e.g. glutamate, serotonin, noradrenaline and tachykinins)[18]. A vast array of experimental NOP ligands are available of both peptide/non-peptide and agonist/antagonist nature. Of relevance are the peptide and non-peptide antagonists UFP-101 and J113397 respectively.[18]

There are a growing number of *in vitro* and *in vivo* studies showing that; (1) N/OFQ-NOP expression in cells of the immune system enables immunomodulation, and (2) N/OFQ-NOP has important cardiovascular modulatory effects. Notably there is growing evidence of the involvement of the N/OFQ system

in sepsis and the inflammatory response. In the following sections this evidence is examined and the links between (1) and (2) are made with respect to sepsis.

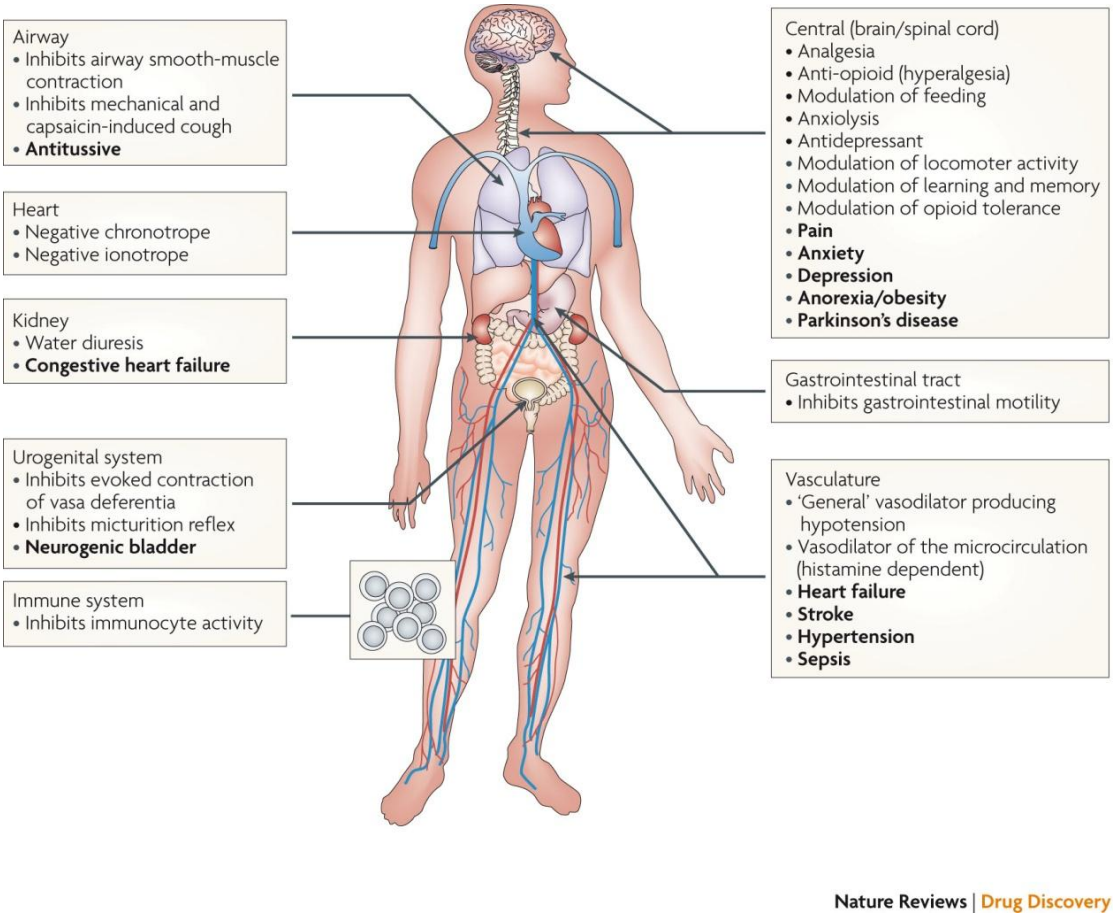

Figure 1-1Schematic representation of the different functions of N/OFQ. Taken from Nature Reviews[18]

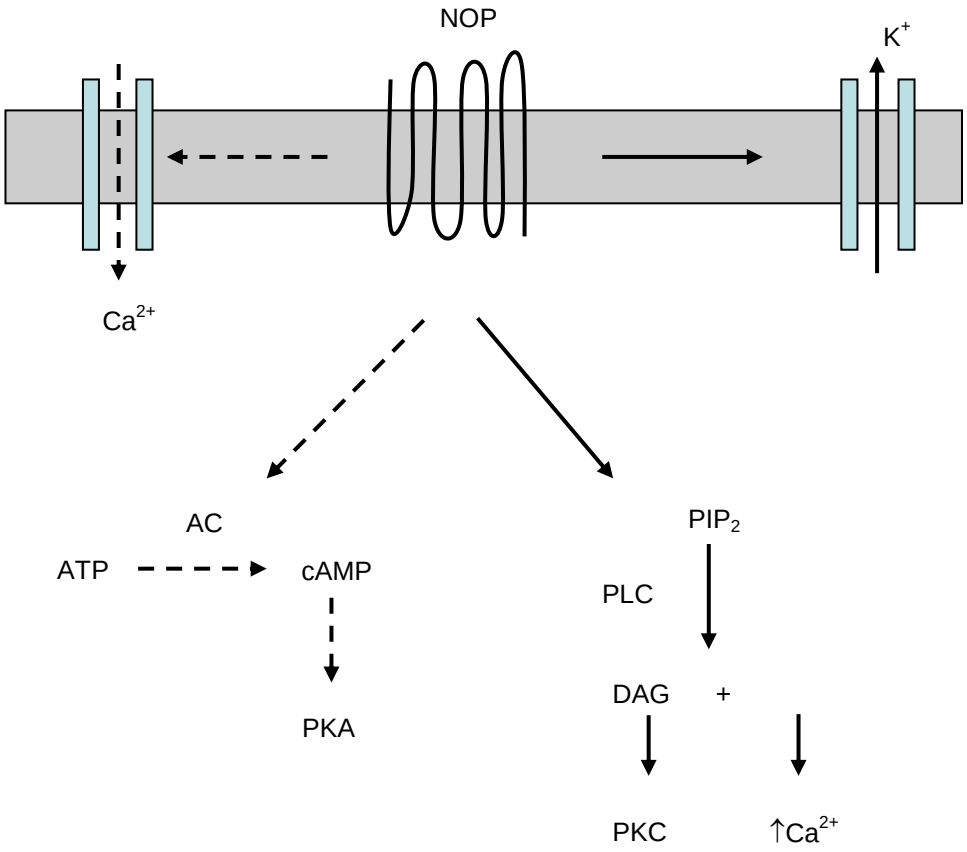

Figure 1-2. Cellular effects of NOP activation. When N/OFQ binds NOP it has a number of actions: 1) inhibits adenylyl cyclase, leading to reduced formation of cAMP; 2) closes the voltage-gated Ca^{2+} channels; and 3) opens the inwardly rectifying K^+ channels. These actions reduce neuronal excitability and neurotransmitter release (glutamate, serotonin, noradrenaline and tachykinins)[18]. NOP also induces activation of mitogen-activated protein kinases (MAPK) and its subtypes: extracellular-signal regulated kinases (ERKs)[13] and c-Jun NH2-terminal kinases (JNK). MAPK regulate various cellular actions such as gene expression, mitosis, cell differentiation, survival and apoptosis.[22] Activation of PLC is controversial. **Block arrows** indicate activation, **dashed arrows** inhibition. NOP: nociceptin/orphaninFQ receptor; AC: adenylyl cyclase; cAMP: cyclic AMP; PKA: protein kinase A; PIP_2: phosphatidylinositol biphosphate; PLC: phospholipase C; DAG: diacylglycerol; IP_3: inositol triphosphate; PKC: protein kinase

1.2 The N/OFQ system in inflammation and sepsis

NOP and N/OFQ precursor (ppN/OFQ) mRNA are found in monocytes, lymphocytes and polymorphonuclear cells[9;14;15] (**Table 1-1**). This is not the case with the other classical opioid receptors (mu;MOP, delta;DOP and kappa;KOP).[16]

Cells studied	Target found	Reference
Lymphocytes & monocytes	NOP	Peluso, 1998[9] Arjomand, 2002[23]
PMN, lymphocytes, monocytes	NOP and ppN/OFQ	Fiset, 2003[14]
PBMC	NOP and ppN/OFQ	Williams, 2007[16] Williams, 2008[15]
PMN	NOP and ppN/OFQ	Young, 2008[17]

Table 1-1 Distribution of the N/OFQ system in human peripheral blood immunocytes. RT-PCR studies. PMN: polymorphonuclear cells. PBMC: peripheral blood mononuclear cells.

In vitro studies show N/OFQ to be produced by immunocytes and to act as an immunomodulator (**Table 1-2**). Its effects are mainly pro-inflammatory, e.g. induction of chemotaxis and proliferation of immune cells. However, some studies showed reduced immune cell proliferation and reduced chemokine production. These differences could be attributed to differences in techniques used to activate immune cells, cell population studied and the response analysed.

Cell	Treatment	Response	Reference
$CD4^+$ T cells	SEB + N/OFQ	↓ cell proliferation with N/OFQ	Easten, 2009[24]
PMN	N/OFQ	Chemotaxis of PMN	Serhan, 2001[25]
Lymphocytes	SEB + N/OFQ	↑cell proliferation with N/OFQ	Waits, 2004[26]
Monocytes	N/OFQ	Monocytes chemotaxis	Trombella, 2005[27]
PMN		No effect on PMN	
PBMC	PHA + N/OFQ	50% of samples showed ↓cell proliferation 50% no effect.	Peluso, 2001[28]
Rat splenocytes	LPS, Con A, IL-1β, TNFα, dexamethasone	↑N/OFQ production	Miller, 2007[29]
Monocytes $CD14^+$	N/OFQ and LPS + N/OFQ	↓ production of chemokines by monocytes	Kaminsky, 2008[30]

Table 1-2 In vitro studies of N/OFQ in volunteer and animal samples. Different cell populations were treated with either N/OFQ or toxins and their response analysed. SEB: Staphylococcal Enterotoxin B. LPS: Lipopolysacharide. ConA: ConvalinA. IL-1β: Interleukin 1β. TNFα: Tumour Necrosis Factor. PHA: phytohaemagglutinin.

In vivo various animal studies have been conducted in septic and non-septic animals, **Table 1-3** . Carvalho and colleagues[31] using a caecal ligation/perforation model of sepsis found that administration of parenteral N/OFQ in rats exacerbated the inflammatory process and increased mortality. Animals treated with N/OFQ had 100% mortality, compared to 50% in those treated with NOP antagonist UFP-101 and to 70% in the control untreated group. N/OFQ treatment also increased plasma concentrations of TNFα and IL-1β. In addition using anaesthetised (but non-septic) rats, Brookes and colleagues[21] showed that N/OFQ produced an inflammatory response composed of vasodilatation, macromolecular leak and leucocyte adhesion in mesenteric vessels.

Conversely, intracerebroventricular administration of N/OFQ led to reduced cytokine production by peritoneal macrophages in rats undergoing exploratory laparotomy.[32] It is possible that there is a difference in the immune response to N/OFQ between peripheral vs central administration and this is an avenue for further investigation.

Model	Intervention	Observations	Reference
Anaesthetised rats	iv N/OFQ	On mesenteric vessels: leucocyte rolling & adhesion, ↑macromolecular leak, ↓blood flow vasodilatation	Brookes, 2007[21]
Septic rats (CLP)	Group 1: sc N/OFQ Group 2: UFP-101 Group 3: Control	Group 1 mortality 100% Group 2 mortality 50% Group 3 mortality 70%	Carvalho, 2008[31]
Rats undergoing exploratory laparotomy	icv N/OFQ	↓production of IL-1 and TNFα by peritoneal macrophages.	Zhao, 2002[32]

Table 1-3 *In vivo* animal studies showing the response to N/OFQ administration. iv: intravenous. sc: subcutaneous. icv: intracerebroventricular. CLP: caecal ligation and perforation. UFP-101: NOP antagonist.

There are few data in humans and to date only two observational studies in septic patients have been published,[33;34] **Table 1-4**. In a small study of 21 critically ill patients admitted to ICU with a diagnosis of sepsis, our research group measured plasma N/OFQ concentrations over 4 consecutive days. Plasma concentrations of N/OFQ on the day of admission to ICU were higher in patients who subsequently died (n=4) compared to those who survived (n=17) (median [IQR] concentrations

3.0 [2.5-5.0] and 1.0 [1.0-2.5] pg ml^{-1} respectively).[33] It is worth noting that these concentrations were lower overall than those reported in the literature from healthy control patients (5 – 15pg.ml^{-1}). Stamer and colleagues[34] recently reported reduced ppN/OFQ mRNA expression in peripheral blood cells in 18 critically ill patients with sepsis, and increased NOP mRNA expression in non-survivors of sepsis, compared with healthy controls. More data are required to confirm these findings

.

Population studied	Response studied	Observations	Reference
21 ICU septic patients	Plasma concentrations of N/OFQ	Plasma N/OFQ concentrations higher in patients who died (n=4) compared to survivors (n=17)	Williams, 2008[33]
18 ICU patients with severe sepsis	NOP and ppN/OFQ mRNA expression in peripheral blood cells	↑NOP mRNA in septic patients who died (n=9) ↓ppN/OFQ mRNA in septic patients compared to controls.	Stamer, 2011[34]

Table 1-4 Human studies of the N/OFQ system during sepsis.

Further supporting evidence for the role of N/OFQ in the inflammatory response comes from gene NOP knockout ($NOP^{-/-}$) mice where the gene for NOP is absent. Kato *et al*[35] induced colitis in mice by giving oral dextran sulfate sodium (DSS). They compared wild-type mice (non-genetically modified, $NOP^{+/+}$) to $NOP^{-/-}$ mice.

Administration of DSS caused bloody diarrhoea in the $NOP^{+/+}$ group compared to normal stools in the $NOP^{-/-}$ group. On histological examination, the colon of $NOP^{+/+}$ mice had crypt distortion and increased numbers of lymphocytes, macrophages and neutrophils (evidence of colitis), compared to normal crypts and reduced numbers of inflammatory cells in the $NOP^{-/-}$ group. This demonstrated that the absence of NOP significantly reduced the inflammatory response to a known pro-inflammatory stimulus (DSS).

1.3 Pain control by N/OFQ during inflammation and the neuroimmune axis

Pain is one of the cardinal features of inflammation and is mediated by prostaglandins, substance P, histamine and other substances.

Endogenous opioids provide analgesia by increasing the number of opioid receptors and the availability of endogenous opioid peptides at the site of inflammation. Opioid receptors are up-regulated in the terminal nerve endings at sites of inflammation; this can be attributed to increased synthesis of receptors in the dorsal root ganglia[36] and increased intra-axonal transport of receptors to the terminal nerve endings (a process mediated by IL-1β).[37] The highest concentration of opioid peptides available at the inflammatory site comes from lymphocytes, monocytes, PMN and macrophages. These immunocytes, during inflammation, have an increased expression of β-endorphin, met-enkephalin and dynorphin-A which is stimulated by endotoxins, viruses, IL-1 and corticotropin releasing hormone (CRH), (**Figure 1-3**). When leucocytes migrate into the inflamed tissue they release these endogenous opioids.[38;39] This mechanism of analgesia by

endogenous opioids acting at peripheral sites has been suggested by Stein and others as being part of a physiological neuroimmune axis.[40]

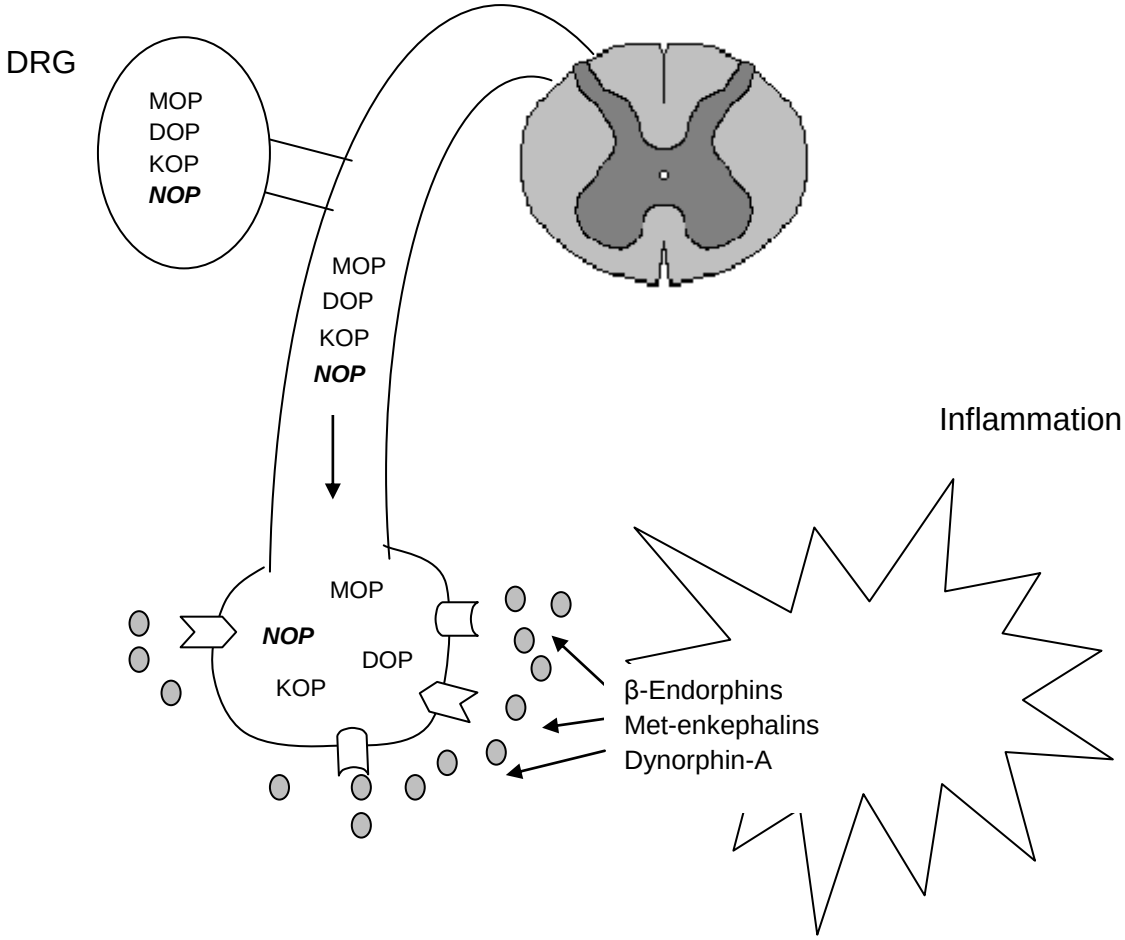

Figure 1-3 Schematic representation of the neuroimmune axis. Classical opioid receptors (MOP: mu; DOP: delta and KOP: kappa) are up-regulated in the terminal nerve endings at sites of inflammation due to increased synthesis in the dorsal root ganglia and increased intra-axonal transport to the terminal nerve endings. Immunocytes at the inflammatory site have increased expression of β-endorphin, met-enkephalin and dynorphin-A.DRG, dorsal root ganglion.

NOP has a similar distribution to classical opioid receptors in the dorsal root ganglion,[41] neuronal tissue,[42] and is also present in leucocytes.[9] Agostini *et al*[43] examined the anti-nociceptive effect of N/OFQ in an animal model of bowel inflammation comparing peripheral and centrally administered N/OFQ. Intraperitoneal administration of N/OFQ reduced the painful response to colorectal distension in rats with induced colitis; in contrast there was no effect when N/OFQ was administered via the intracerebroventricular route. Administration of the N/OFQ antagonist UFP-101 increased the painful response to colorectal distension and antagonised the anti-nociceptive effect of N/OFQ. An increased number of cells positive for NOP and myeloperoxidase activity (a marker of PMN granulation) were observed in a segment of distal colon, which confirmed the infiltration of immunocytes into the inflamed tissue. These findings support the hypothesis that N/OFQ may act peripherally as an analgesic at sites of inflammation. More classical modulation by N/OFQ of the pain response is covered extensively by Zeilhofer *et al*[44] and Lambert *et al*[18] and is represented in **Figure 1-4** .

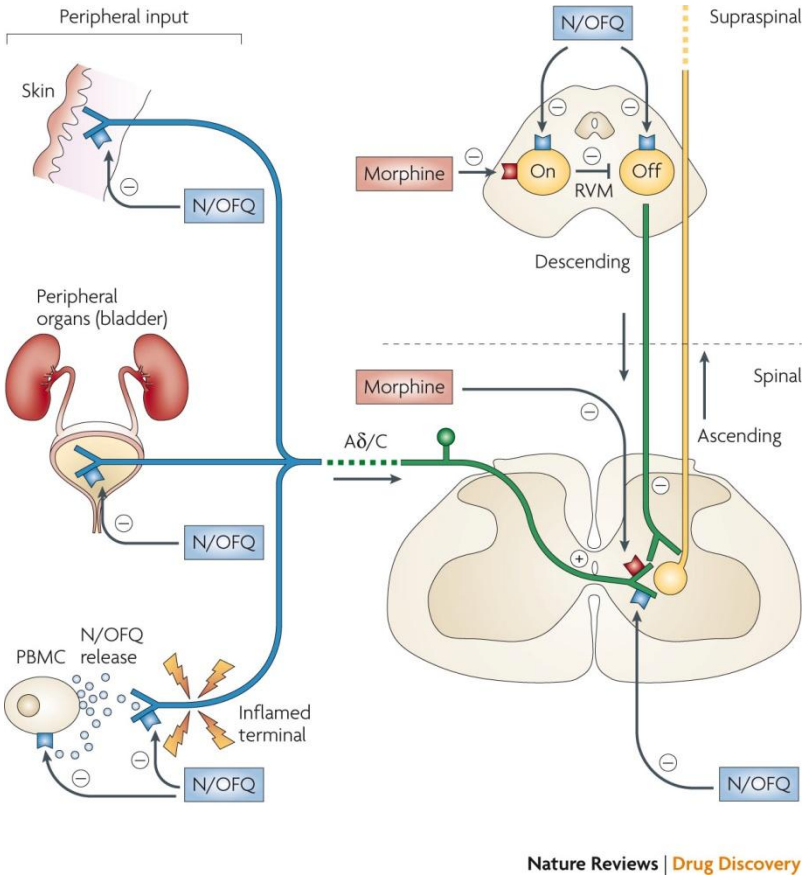

Figure 1-4 Schematic representation of pain modulation by N/OFQ. N/OFQ has opposite supraspinal and peripheral actions.

At supraspinal level N/OFQ causes hyperalgesia/anti-opioid effect by inhibiting both On- and Off- cells in the rostral ventromedial medulla (RVM) (On- and Off-cells are two classes of pain-modulating neurons. On-cells increase firing before the nociceptive reflexes and inhibit Off-cells. Off-cells stop the firing before the nociceptive reflexes occur and are activated by disinhibition).[45] This counteracts the analgesic effect of morphine which inhibits the On-cells and this in turn disinnhibits the Off-cells, leading to analgesia.

At peripheral level, N/OFQ inhibits nociceptive afferent inflow in tissues such as skin, bladder and probably at inflammation sites as described above.

1.4 N/OFQ and the Hypothalamic-Pituitary-Adrenal (HPA) axis

The hypothalamus is involved in regulation of the stress response through the production of steroids and in particular cortisol via the feedback system of the HPA axis. Corticotropin releasing hormone (CRH), produced in the hypothalamus, controls the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn regulates the release of glucocorticoids (mainly cortisol) from the adrenal cortex.[46] Cortisol facilitates the normal response of catecholamines (adrenaline and noradrenaline), angiotensin and vasopressin, up-regulates receptor expression and has anti-inflammatory effects: 1) reduction of the function and number of lymphocytes, monocytes, neutrophils and eosinophils at the site of inflammation; 2) reduction of macrophage adhesion to the endothelium and 3) reduction of IL-2 production from macrophages.[47]

N/OFQ activates the HPA axis under resting and mild stress conditions. Intracerebroventricular injection of N/OFQ in rats led to increased plasma corticosterone[48] and ACTH concentrations[49] for up to 30 minutes under resting conditions. N/OFQ administered to rats under mild stress, enhanced the raised ACTH response to stress and prolonged the higher concentrations of corticosterone. Under a more stressful stimulus like restraint, intracerebroventricular N/OFQ did not affect the already increased plasma concentrations of ACTH or corticosterone.[49] Leggett *et al*[50] also observed

increased CRH mRNA expression in the paraventricular nucleus of the hypothalamus in rats after intracerebroventricular N/OFQ administration.

These data show the potential effects of N/OFQ on HPA axis activity. Whether these effects occur during an episode of systemic inflammation and lead to maintenance of plasma hormone concentrations or to an immunosuppressant effect has yet to be determined. Further data are required.

1.5 Effects of N/OFQ in the cardiovascular system

Activation of the immune system during inflammation and sepsis can cause significant cardiovascular changes leading to hypotension, hypo-perfusion and subsequent multi-organ failure. Endogenous and exogenous opioids have important cardiovascular effects.[51] Similarly, N/OFQ causes hypotension and bradycardia when administered intravenously and intracerebroventricularly, as demonstrated by various *in vitro* and *in vivo* animal studies and this has been covered extensively in previous reviews.[18;52] Different mechanisms of action have been proposed and here we concentrate on the data which suggest an immuno-vascular link.

1.5.1 Centrally mediated cardiovascular effects

The central nervous system contributes to the cardiovascular response during inflammation via the hypothalamic-pituitary-axis, the sympathetic nervous system and the cholinergic anti-inflammatory pathway.[53] Over the past fifteen years it has been found that NOP, N/OFQ and ppN/OFQ are present in areas of animal and human brains associated with cardiovascular regulation. ppNOF/Q mRNA has been isolated from brainstem in mice;[54;55] NOP has been identified in the hypothalamus[56] and brainstem[57] of human foetus and adult rats; and N/OFQ is found in rat and human (neonatal deaths or foetal losses) brainstem[57].

In vivo, administration of intracerebroventricular N/OFQ in mice caused a dose-dependent drop in blood pressure and heart rate.[19;20] These cardiovascular changes had a rapid onset (< 1 minute) with a peak effect at 20 mins, and duration of 40 – 50 mins. Pre-treatment with intracerebroventricular UFP-101, abolished the cardiovascular response to N/OFQ.[19]

Notably the administration of intracerebroventricular UFP-101 alone in mice, also led to significant bradycardia for up to 2 hrs.[19] A similar finding was also reported in conscious rats.[58] This may be due to a weak partial agonist effect of centrally administered UFP-101 when NOP expression is high. N/OFQ has also been injected in more specific areas of the brain thought to be involved with the regulation of the cardiovascular system: the rostral ventrolateral medulla (RVLM) and the paraventricular nucleus of the hypothalamus. Direct administration into

these areas caused dose-dependent bradycardia and hypotension in anaesthetised rats.[59] Using comparable doses to those used in the intracerebroventricular studies, the cardiovascular effects also had a rapid onset and similar duration of 30 to 60 mins. Pre-treatment with the NOP antagonist [Phe^{1}ψ(CH_2-NH)Gly^2]N/OFQ(1–13)NH_2 blocked the cardiovascular response to N/OFQ. This NOP antagonist peptide was originally described as an antagonist in the vas deferens where expression of NOP is low,[60] but subsequent work has revealed partial agonist activity.[61;62]

Burmeister and colleagues[20] compared the effects of intracerebroventricular N/OFQ in wild-type $NOP^{+/+}$ and NOP knockout $NOP^{-/-}$ mice. Basal values for mean arterial pressure and heart rate were similar in both groups. On administration of intracerebroventricular N/OFQ, significant hypotension and bradycardia occurred in $NOP^{+/+}$ mice, in contrast to no cardiovascular changes in $NOP^{-/-}$ mice. These data show the cardiodepressant effect of N/OFQ to be mediated by NOP when N/OFQ is administered centrally in healthy animals. It is unknown whether the expression of the N/OFQ system in the brain is altered during sepsis; however, current evidence suggests this may be an option worthy of study.

1.5.2 Peripherally mediated cardiovascular effects

Multiple mechanisms are involved in the cardiovascular response that occurs during inflammation and sepsis. The N/OFQ system is present in the immune cells

and activated as a response to inflammatory stimulus. Activation of immunocytes and the release of N/OFQ possibly have an effect on vascular reactivity.[63] N/OFQ causes vasodilatation and increased capillary leak from mesenteric vessels that could be a potential target during inflammation.[21]

The peripheral actions of N/OFQ have been noted by the cardio-depressor response observed after intravenous administration of N/OFQ in anaesthetised rats,[21;64] guinea pigs[65] and conscious mice[66]. These studies used similar doses of N/OFQ, between 10 and 100 $nmol.kg^{-1}$, and demonstrated dose-dependent bradycardia and hypotension of nearly 30%,[65] which lasted around 10 minutes. These cardiovascular effects were antagonised by pre-treatment with UFP-101. Based on evidence from animal studies (**Figure 1-5**) there are 4 potential mechanisms for the peripheral effects of N/OFQ:

1) pre-synaptic inhibition of noradrenergic neurotransmission;

2) direct arterial vasodilatation with reduced blood flow;

3) histamine mediated,

4) via the parasympathetic nervous system (PNS).

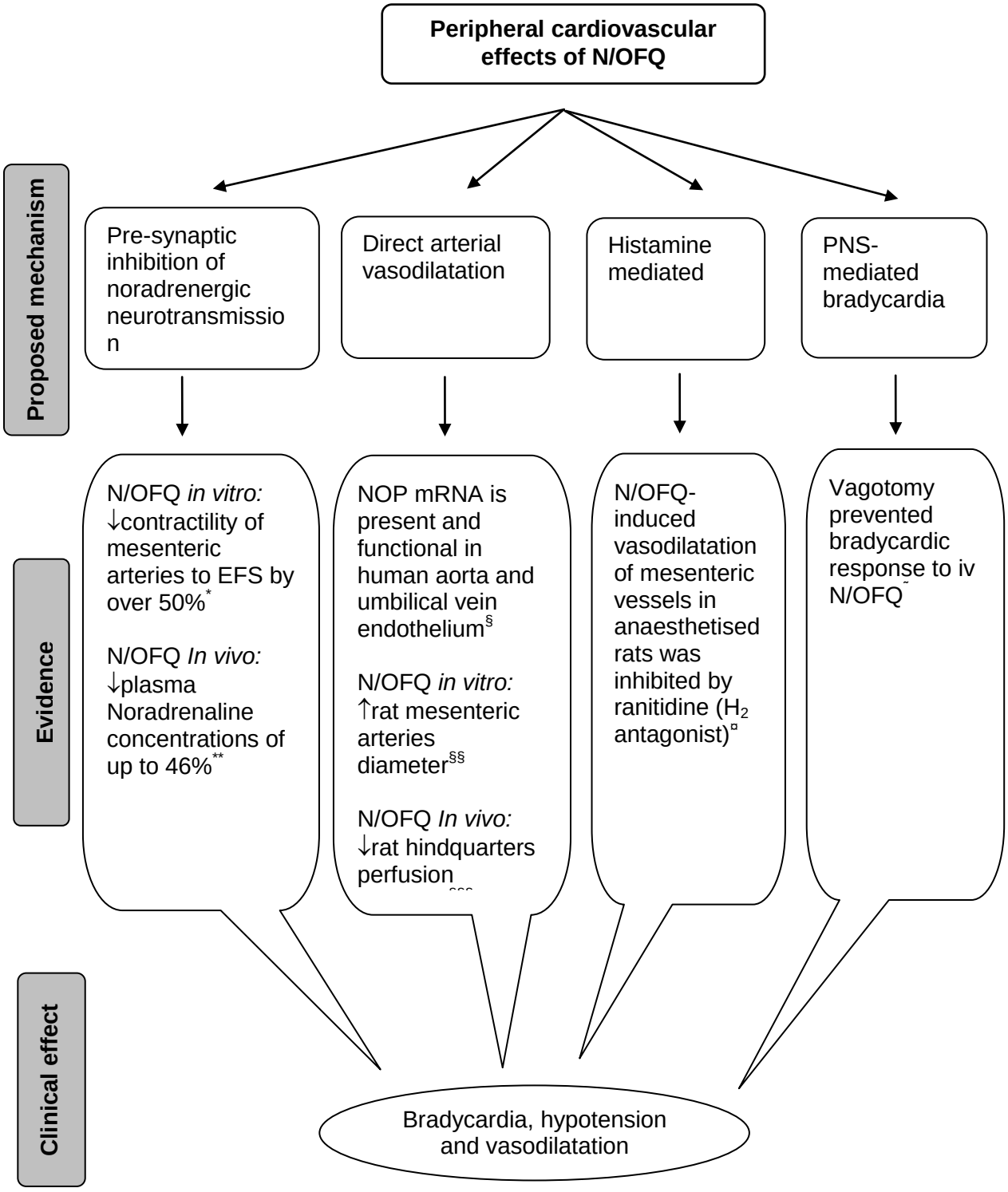

Figure 1-5 Suggested peripherally mediated mechanisms of action of N/OFQ in the cardiovascular system.

PNS: parasympathetic nervous system. EFS: Electrical field stimulation. NA: Noradrenaline.

* In rat mesenteric arteries[67] and anococcygeal smooth muscle[68]. Pre-contracted arteries with noradrenaline were not antagonised by N/OFQ.

**In anaesthetised guinea pigs[65]

§ Endothelial cells were exposed to N/OFQ and showed some functionality by activating protein kinases.[13]

§§ Rat mesenteric arteries were pre-constricted with a thromboxane A2 agonist.[69;70] N/OFQ caused a dose-dependant increase in vessel diameter, which was not affected by removal of endothelium, exposure to nitric oxide synthase inhibitor, atropine or phentolamine.

§§§ A response that was not altered by exposure to nitric oxide synthase inhibitor.[70]

¤Brookes[21]

˜N/OFQ iv 10, 30 and 100 nmol.kg^{-1}, caused transient bradycardia and hypotension in anaesthetised rats. Post- bilateral vagotomy, the animals had less bradycardia to N/OFQ but the hypotensive response did not change significantly.[64]

1.6 Leucocytes, PAMPs, DAMPs and CARS

The human body responds to trauma, injury or infection by mounting an inflammatory response. The immune system defends the organism via innate and acquired immune responses, and is responsible for the degree of local or systemic inflammation. Leucocytes are part of the innate immune system and in normal circumstances count varies between 4 – 11 x 10^9/l. Leucocytes are classified as neutrophils (40 – 75%), lymphocytes (20 – 50%), monocytes (2- 10%), eosinophils (1- 6%) and basophils (<1%).[71] Lymphocytes and monocytes are mononuclear cells (PBMC) whilst neutrophils, eosinophils and basophils are polymorphonuclear cells (PMN), (**Figure 1-6**). Of these, the neutrophils are the most important in the pathogenesis of the inflammatory response.

Neutrophilic leucocytosis, occurs commonly in bacterial infection, stress, smoking, pregnancy and following exercise. Lymphocytic leucocytosis, is more commonly seen in viral infections, e.g. infectious mononucleosis, pertussis, or in lymphocytic leukemias.

The response to sepsis evolves over time as a dynamic and complex process. Activated neutrophils express toll-like receptors (TLRs) which recognize specific molecules produced by microbial pathogens (pathogen-associated molecular patterns (PAMPs). Lipo-polysaccharides, peptidoglycans, bacterial lipoprotein, and bacterial RNA are examples of PAMPs which are only produced by bacteria. Activation of TLRs cause activation of several signalling pathways amongst which are the release of pro-inflammatory cytokines (IL-1β, TNFα, leukotrienes),

antimicrobial peptides, and reactive oxygen species.[72] When host cell colonisation and damage occurs, there is production of 'alarmins' or 'danger-associated molecular patterns' (DAMPs) molecules which, in the same way as PAMPs, activate TLRs. This cross-reactivity of TLR to both PAMPS and DAMPS worsen the inflammatory response.(72, Bianchi 2007).

Persistent exposure to PAMPs reduces TLRs signalling and therefore reduces production of pro-inflammatory cytokines with up-regulation of TLR inhibitors. The development of a compensatory anti-inflammatory response syndrome (CARS), which seems to occur simultaneously with the pro-inflammatory response, is characterized by the release of cytokines such as IL-10, IL-1 receptor antagonist (IL-1ra), IL-4, prostaglandins and immuno-modulatory hormones.[73] Serum concentrations of both pro- and anti-inflammatory cytokines are raised early on during sepsis. As sepsis progresses an anti-inflammatory immunosuppressive state develops with opportunistic secondary infections that persist even after the pathogen has been cleared. Morbidity and mortality during SIRS and sepsis is likely to be due to both, an excessive pro-inflammatory response to tissue damage (infective or non-infective) and the opposing compensatory anti-inflammatory response syndrome. These are the basis of sepsis and multi-organ failure.[74;75]

We have chosen to study the expression and changes of the N/OFQ system during inflammation and sepsis in polymorphonuclear cells.

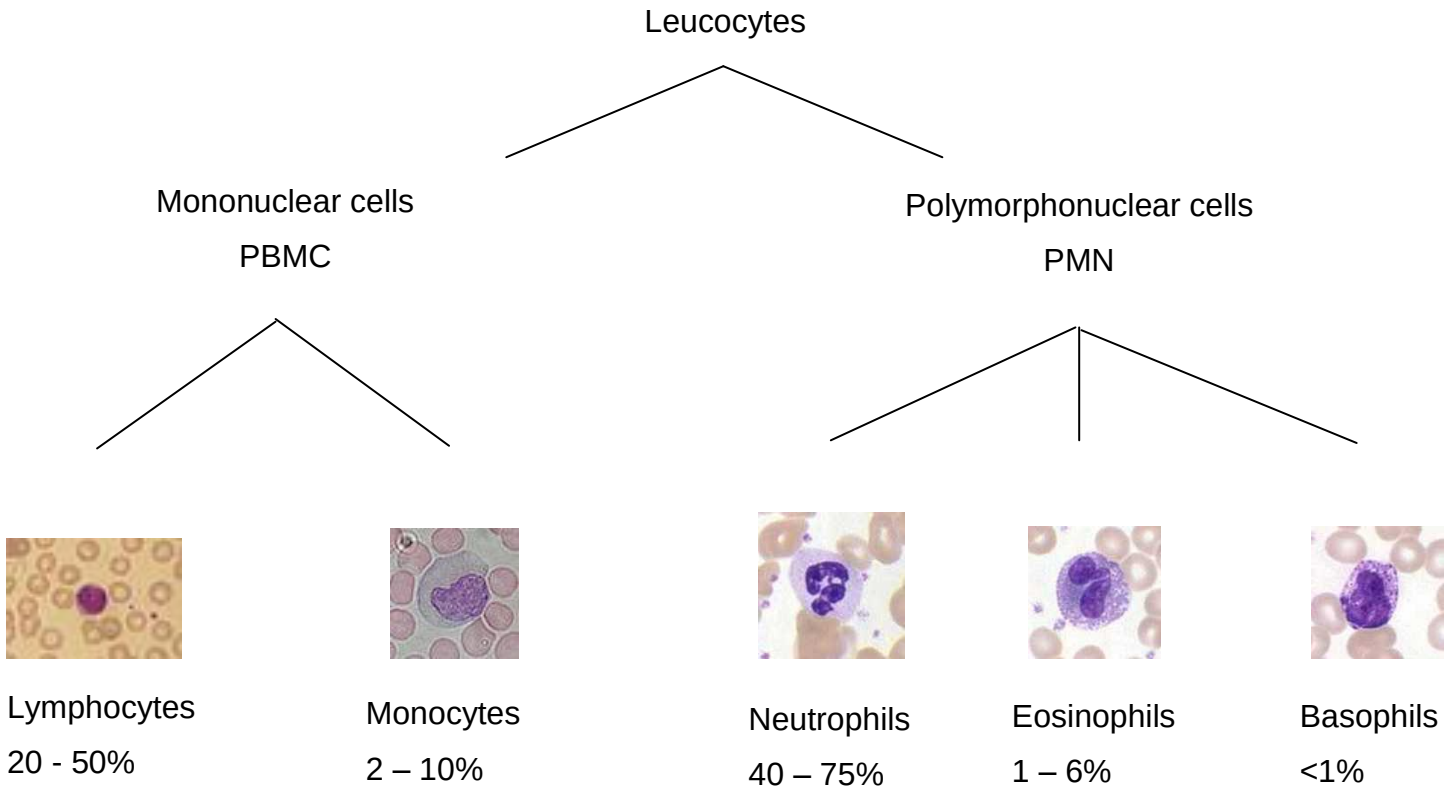

Figure 1-6 Schematic diagram of leucocyte classification. PBMC, peripheral blood mononuclear cell. Pictures taken from http://en.wikipedia.org/wiki/White_blood_cell - Accessed on 30 April 2012.

1.7 Inflammatory markers

Inflammation, tissue injury and sepsis are some of the conditions that trigger a pathophysiological cascade involving release of acute phase proteins and cytokines. Acute phase proteins either increase or decrease (positive or negative response) by at least 25% during an inflammatory response. Positive acute phase proteins include CRP (C-reactive protein), amyloid, fibrinogen, alpha-1-antitrypsin, IL-1Ra (receptor antagonist), haptoglobin, and ferritin. Negative acute phase proteins include albumin, alpha-foetoprotein and transferrin.[76] The erythrocyte sedimentation rate (ESR) is a non-protein marker of inflammation that reflects the presence of acute phase proteins by increased plasma viscosity. Plasma concentrations of acute phase proteins and cytokines often represent the presence of an inflammatory process and its severity. However, they are also affected by chronic conditions, age, sex, and are not specific to a particular disease, and do not change uniformly in all patients with the same condition.[76;77]

Changes of the acute phase proteins occur mainly due to cytokines. Innate immune cells, i.e. macrophages, monocytes, lymphocytes, neutrophils and dendritic cells generate and release various pro- and anti- inflammatory cytokines. Pro-inflammatory cytokines promote the development and maintenance of the inflammatory response and include IL-1, IL-6, IL-8, IL-12, IL-18, TNFα, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), and interferon γ (IFN gamma).[78] IL-1, IL-6, TNF-α, and IFNγ are considered to be the main inducers of the inflammatory cascade, they play an important role in initiating other

cytokines that make up the immune response to infection.[76;78] The TNFα receptor has the ability to activate multiple signal transduction pathways and the ability to induce or suppress genes such as those for growth factors, cytokines, transcription factors and acute phase proteins.[79] TNFα is an inflammatory marker during sepsis and has also been a therapeutic target; however, various trials have failed to demonstrate improvement in mortality.[80;81]

Anti-inflammatory cytokines IL-1Ra, IL-4, IL-6, IL-10, IL-11, and IL-13 limit the inflammatory response.[82] IL-10 inhibits the production of TNF-α, IL-6 and IL-8 by monocytes.[78;83] The balance maintained between pro- and anti- inflammatory cytokines determines the severity of the inflammatory response and disease.

Various studies have looked into immunomodulatory therapy *in vitro* and *in vivo* in animal and human studies with disappointing results. Furthermore, animal studies do not necessarily have the same response in humans. An animal model of sepsis by caecal ligation and perforation (CLP) showed inhibition of IL-6, myeloperoxidase, and reduced mortality when treated with JTE-607, an inhibitor of cytokine (IL-6, IL-8, TNFα, IL-1β, IL-10, IL-1ra, and GM-CSF) production.[84] In a human model of endotoxemia, JTE-607 decreased endotoxin-induced IL-10 and IL-1ra production.[85] Other immunomodulators that have been researched are recombinant human IL-1ra, which did not improve mortality of patients with severe sepsis in a multi-centre clinical trial,[86] and toll-like receptor 4 antagonist, eritoran tetrasodium, did not show changes in mortality compared to placebo in 300 critically ill patients with severe sepsis.[87] The multiple pathways involved in the pathophysiology of sepsis, and redundant signalling pathways may explain the difficulties in developing immunomodulatory therapy in critically ill patients with sepsis.

PMN cells when activated, also release myeloperoxidase (MPO),[88] matrix-metalloproteinases (MMPs), PMN elastases and oxygen-free radicals.[89] Myeloperoxidase (MPO) is the most abundant protein in PMN cells,[88] stored in the azurophilic granules, and is also found to a lesser extent in monocytes. The infiltration of PMN cells into inflamed tissues can be assessed by determining MPO activity. MPO contributes to PMN cell recruitment, killing of microorganisms engulfed by neutrophils and monocytes, and can be released outside the cell and cause damage to an extracellular target.[90] MPO plasma concentration is increased in conditions such as SIRS, sepsis,[91] and acute myocardial infarction[92]

Procalcitonin (PCT) is another marker of inflammation and sepsis that has been shown to be superior to CRP in the diagnosis of sepsis.[93;94] However, there are conflicting views in the accuracy of PCT in differentiating infective and non-infective SIRS, and its ability to determine prognosis and mortality from sepsis.[93;95-97]

1.8 Inflammatory response during Cardiopulmonary bypass (CPB)

CPB during cardiac surgery is used to maintain perfusion to the brain and other vital organs, provide cardiac and pulmonary protection, and maintain thermoregulation for organ protection while providing appropriate conditions to the surgeons to perform cardiac surgery. The use of an extracorporeal circuit in CPB involves non-pulsatile flow, contact of blood with foreign surfaces, changes in venous pressure and core temperature and ischemia-reperfusion injury. All of

these trigger the release of inflammatory cytokines, activation of endothelial cells, complement, kinin and coagulation cascades and activation of the innate immune system. Activated PMN cells release proteolytic enzymes: MPO (myeloperoxidase), MMPs (matrix-metalloproteinases), PMN elastases and oxygen-free radicals, all of which promote organ dysfunction. PMN cell activation is enhanced by raised cytokines.[88;89]

Non-pulsatile flow, ischemia-reperfusion injury and alterations in blood flow have potential deleterious effects that can lead to myocardial injury, lung injury, renal dysfunction, neurologic dysfunction, bleeding and coagulopathy.[98-100]

Extensive research has been conducted in patients undergoing cardiac surgery under CPB in order to decrease and understand the inflammatory response to a non-infective inflammatory stimulus.[98;101] Different CPB circuits, pharmacological agents (glucocorticoids, protease inhibitors, phosphodiesterase inhibitors, antioxidants, complement inhibition and monoclonal antibodies) and surgical techniques are some of the strategies that have been investigated. Further details on the inflammatory response to CPB are described in Chapter 3: The effect of Cardiac surgery under cardiopulmonary bypass on the N/OFQ system, and is covered by Paparella and colleagues.[101]

1.9 Summary

The N/OFQ system is present in multiple organ systems and has been attributed different roles in pain control, anxiety, depression, drug abuse and heart failure.[18] Because of the location of NOP and N/OFQ in immune cells, a number of studies have examined its involvement in inflammatory responses,

and specifically how it affects the immune response, the cardiovascular system and the HPA axis.

The evidence available that the N/OFQ-NOP system has a role in sepsis-inflammation is largely based on *in vitro* and *in vivo* animal studies; with only two observational studies in human patients with sepsis.[33;34] One of these was our own and involved a very small number of patients. However, based on these studies, one conclusion from these data is that the expression of the N/OFQ system by leucocytes increases during sepsis. Circulating leucocytes could then become N/OFQ delivery vehicles releasing N/OFQ at the site of inflammation which may produce systemic hypotension and organ dysfunction. Indeed, this may form an immuno-vascular axis. As NOP activation is chemotactic the upregulation of this receptor in sepsis may contribute (possibly via auto-activation) to the regulation of the inflammatory response. However, these hypotheses require rigorous experimental validation as it remains unclear how N/OFQ and NOP alter in patients with an ongoing inflammatory process or systemic sepsis and whether the changes would correlate with pathophysiological effects. When we have this information it should be possible to design appropriate clinical studies to evaluate NOP ligands in sepsis. Based on existing data[31;33] we would predict that NOP antagonists might be useful in this situation as they might 1) reduce the systemic effects of leucocyte delivered peptide and 2) reduce the chemotactic response and ongoing inflammation.

1.10 Aims

The aims of the studies described in this thesis were:

1. To study mRNA expression of the N/OFQ system in polymorphonuclear cells during inflammation and sepsis.

2. To study the plasma concentrations of N/OFQ during inflammation and sepsis.

3. To analyze the relationship between 1) and 2), and with physiological and biochemical markers of inflammation and sepsis (e.g. white cell count, CRP, and cytokines).

4. To determine if changes in the N/OFQ system during inflammation and sepsis relate to patient's morbidity and outcome.

The thesis is based on detailed analysis of clinical data and laboratory analysis from two observational studies. The first involved patients having an inflammatory response unrelated to sepsis, and the second recruited patients with severe systemic sepsis admitted to ICU:

Study 1: Patients undergoing cardiac surgery under cardio-pulmonary bypass (CPB, as a non-septic model of inflammation without infection). Blood samples for plasma and polymorphonuclear cell analysis were obtained at 3 stages: 1) Baseline, before surgery, 2) at 3 hours post-CPB, and 3) at 18 – 24 hours post-CPB.

Study 2: Patients admitted to the Intensive Care Unit with a clinical diagnosis of sepsis. Blood samples were collected within the first 24h of ICU admission, and

a second blood sample on the following day. A third blood sample, where possible, was collected once patients recovered from sepsis, and this served as a within-patient control sample.

In the absence of any usable antibodies for Western blot or Immunocytochemistry NOP expression is tracked using quantitative real time PCR.

1.11 Hypothesis

The expression of NOP and ppN/OFQ mRNA by polymorphonuclear cells increases during systemic inflammation and sepsis. Plasma concentrations of N/OFQ are also increased as these are likely to originate from polymorphonuclear cells.

2 Methods

We have investigated the change of the nociceptin (N/OFQ) system in plasma and PMN cells in patients undergoing cardiac surgery under cardiopulmonary bypass and in critically ill patients with sepsis. Plasma concentrations of selected cytokines were also analysed. In addition to work in clinic we have used the following laboratory methods:

- Gradient Polymorphprep® for PMN cells extraction
- Cell counter and flow cytometry (FCM) to confirm purity of PMN cell extraction
- Polymerase Chain Reaction (RT-qPCR) to study the gene expression of NOP and ppN/OFQ (N/OFQ precursor) by PMN cells.
- Radioimmunoassay (RIA) to determine plasma N/OFQ concentrations.
- ELISA to determine plasma concentrations of cytokines.

2.1 Blood collection

Blood collection procedure was the same for both cardiac surgery and sepsis studies. A total of 22.5 ml whole blood was aspirated from the arterial line (in most of the cases where one was in place, otherwise blood was aspirated from the central venous access). Fifteen ml of blood were drawn into two 7.5 ml EDTA bottles and transferred at room temperature to the laboratory. Polymorphonuclear (PMN) leucocytes were then isolated by a gradient method, within 30 minutes of the sample being taken.

The remaining 7.5 ml blood was drawn into a third EDTA bottle. 150 µl aprotinin (containing 4.5 TIU, 0.6 TIU.ml^{-1}) was immediately added to prevent protein degradation. This sample was transferred on ice to the laboratory and centrifuged at 3,000x g, 4˚C for 15 minutes. The plasma obtained was stored in 1 ml aliquots at -80˚C and used for batch analysis of N/OFQ peptide by radioimmunoassay and of cytokines and MPO by ELISA.

Analysis of inflammatory markers is commonly used in research and there are recommendations for optimal collection, storage and analysis of samples. Most studies suggest that EDTA plasma samples is the optimal way to analyse inflammatory markers. Sample collection and processing should be conducted in a standardised manner. Immediate sample processing is not often possible and therefore storage should be adequate. Samples should be kept cooled during processing as some inflammatory markers can degrade or increase at room temperature; TNFα seems to degrade at room temperature after 4 - 6 hours.[102] When samples are stored over a long time degradation becomes an issue. The temperature in a mechanical -80C freezer can vary depending mainly on the location within the freezer (measured temperature can change between -90 to -43.5). Assessment for degradation is difficult and different results can be attributed to other variables like changes in the laboratory assay over time, intra- and inter-observer variations. Generally, it is recommended to store EDTA plasma samples for analysis of inflammatory markers at -70C or lower and to avoid freeze-thaw cycles as some inflammatory markers are more sensitive.[102]

2.2 Polymorphonuclear cell extraction

Fifteen ml of whole blood was aspirated from the arterial line into two 7.5 ml EDTA bottles and transferred at room temperature to the laboratory. Polymorphonuclear (PMN) leucocytes were isolated within 30 minutes of sample being taken by a gradient method, Polymorphprep™ (Axis-Shield). Following manufacturer's instructions, 7.5 ml whole blood were carefully layered over 7.5 ml Polymorphprep™ into two 15 ml centrifuge tubes. Samples were centrifuged at 500x g for 30 minutes at 20˚C with 0 deceleration force (brake off) to avoid disturbance of the layers.

After centrifugation, 2 bands of leucocytes were obtained, the top band corresponding to mononuclear cells and the bottom band to polymorphonuclear cells (**Figure 2-1**).

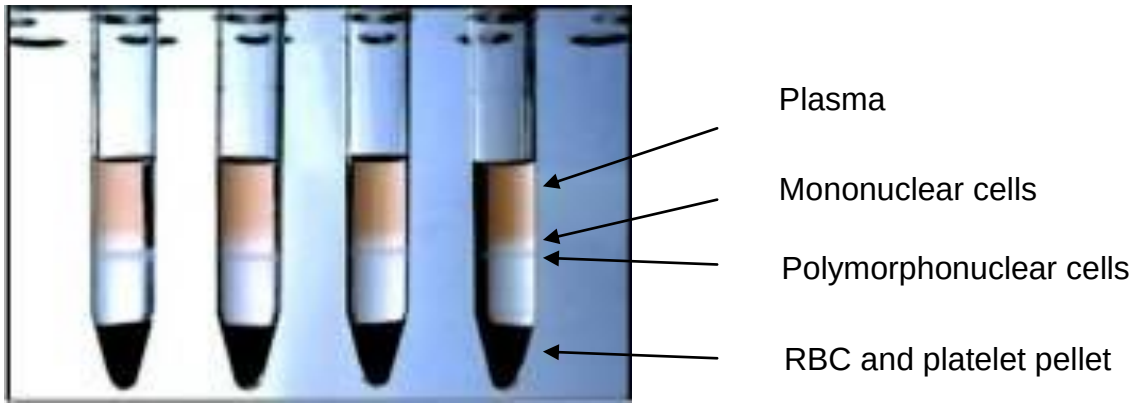

Figure 2-1. Schematic representation of polymorphonuclear leucocyte extraction by gradient method.

The polymorphonuclear band was harvested and Phosphate Buffered Saline (PBS) added in equal volumes for washing. The cells were centrifuged at 500x g for 10 minutes at 20˚C. The supernatant was discarded and the pellet

resuspended in 1 ml of Tri-reagent® (RNA isolation reagent). The samples were stored at -80˚C for batch analysis.

2.3 Cell counter and Flow Cytometry

2.3.1 Theory

To determine the level of contamination of the PMN cell layer extracted using the gradient method, we performed flow cytometry (FCM). 1 volunteer and 2 patient samples were analysed using a Coulter® EPICS XL™.MCL Flow Cytometer.

FCM allows differentiating the type of cells present in a sample according to its physical and/or chemical properties, like size, cytoplasmic granularity and surface antigens. In brief, FCM uses *light scattering* and *fluorescence* by means of lasers to differentiate cell types. For example, *light scattering* is used to differentiate between monocytes, lymphocytes and granulocytes. The cells are made to pass in a single line in a fluid stream and a laser light directed to them. The measurement of light scattered by the cells is performed at two angles, 0˚ or forward scatter and 90˚ or side scatter[103] (**Figure 2-2**). Forward scatter determines the size of the cells, the bigger the cell the larger the light scatter. Light scatter is then represented in voltage. Side scatter determines the complexity and cytoplasmic granularity, granulocytes with higher cytoplasmic granularity produce higher scatter of light than monocytes, which in turn is higher than lymphocytes.[104]

Fluorescence is used to differentiate cell subtypes by staining cells with an appropriate fluorophore-labelled antibody against specific surface antigens

(**Figure 2-3**). For example, in peripheral blood samples CD66a, CD66b, CD66c and CD66d antigens are only expressed in granulocytes. When a laser light hits the fluorophore-labelled antibody a signal is emitted and represented in voltage directly proportional to the amount of labelled antibody. A variety of dyes for antibodies are available, in our experiments we used Fluorescein isothiocyanate (FITC) and R-Phycoerythrin-Cyanine 5 (RPE-Cy5) conjugated dyes.

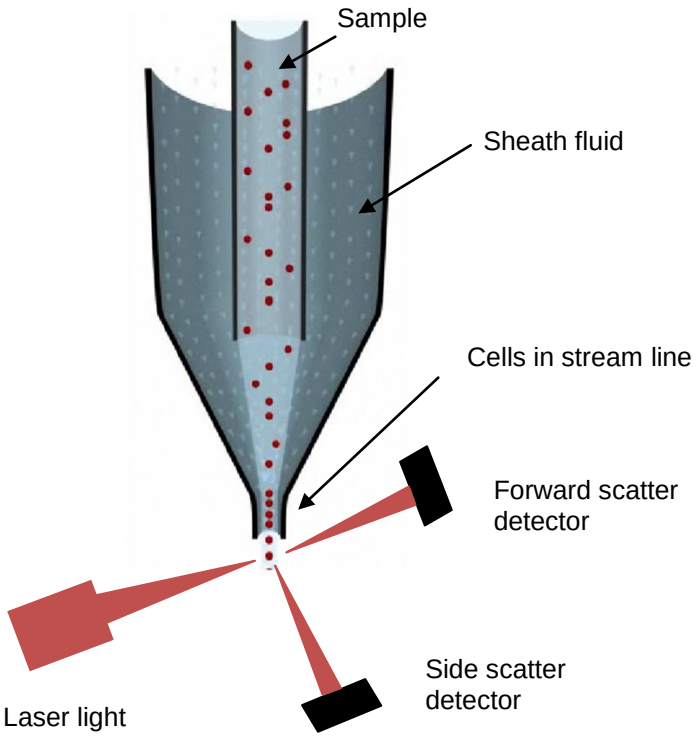

Figure 2-2. Flow cytometry. Schematic representation showing cells passing one at the time with forward and side scatter detectors. Adapted from MRC Clinical Sciences Centre, http://flow.csc.mrc.ac.uk

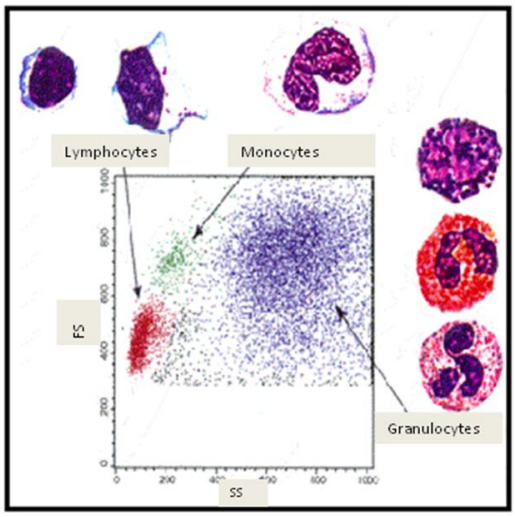

Figure 2-3 Graphical representation of side scatter, forward scatter and fluorescence in flow cytometry.
Taken from http://www.wadsworth.org/cores/immunology/flowp.html

2.3.2 FCM materials

Phosphate buffered saline (PBS), 0.1% sodium azide, 0.1% bovine serum albumin (BSA) and 1% formaldehyde.

Antibodies:

- For mononuclear cells: CD45 and CD45-RPE-Cy5 as negative control.
- For granulocytes: CD66abce (Monoclonal Mouse Anti-Human) and CD66-FITC as negative control.

2.3.3 FCM Method

Harvested PMN cells were washed in an equal volume of PBS, and then cells were centrifuged at 500x g, at 20˚C for 10 minutes. The cell pellet was resuspended in 0.5 ml of PBS. The samples were divided into 3 tubes and washed with PBS + Sodium Azide 0.1% + 0.1% Bovine Serum Albumin (BSA)

and centrifuged at 500x g, at room temperature for 5 minutes. The supernatant was discarded.

To each of the tubes we added the following:

Tube 1: CD45-RPE-Cy5 negative control

Tube 2: CD45 antibody + CD66-FITC negative control

Tube 3: CD45 + CD66abce

The samples were incubated at room temperature for 30 minutes protected from light, and then we added 1% formaldehyde 500 µl. Samples were analysed in a Coulter® EPICS XL™.MCL Flow Cytometer (**Figure 2-4**).

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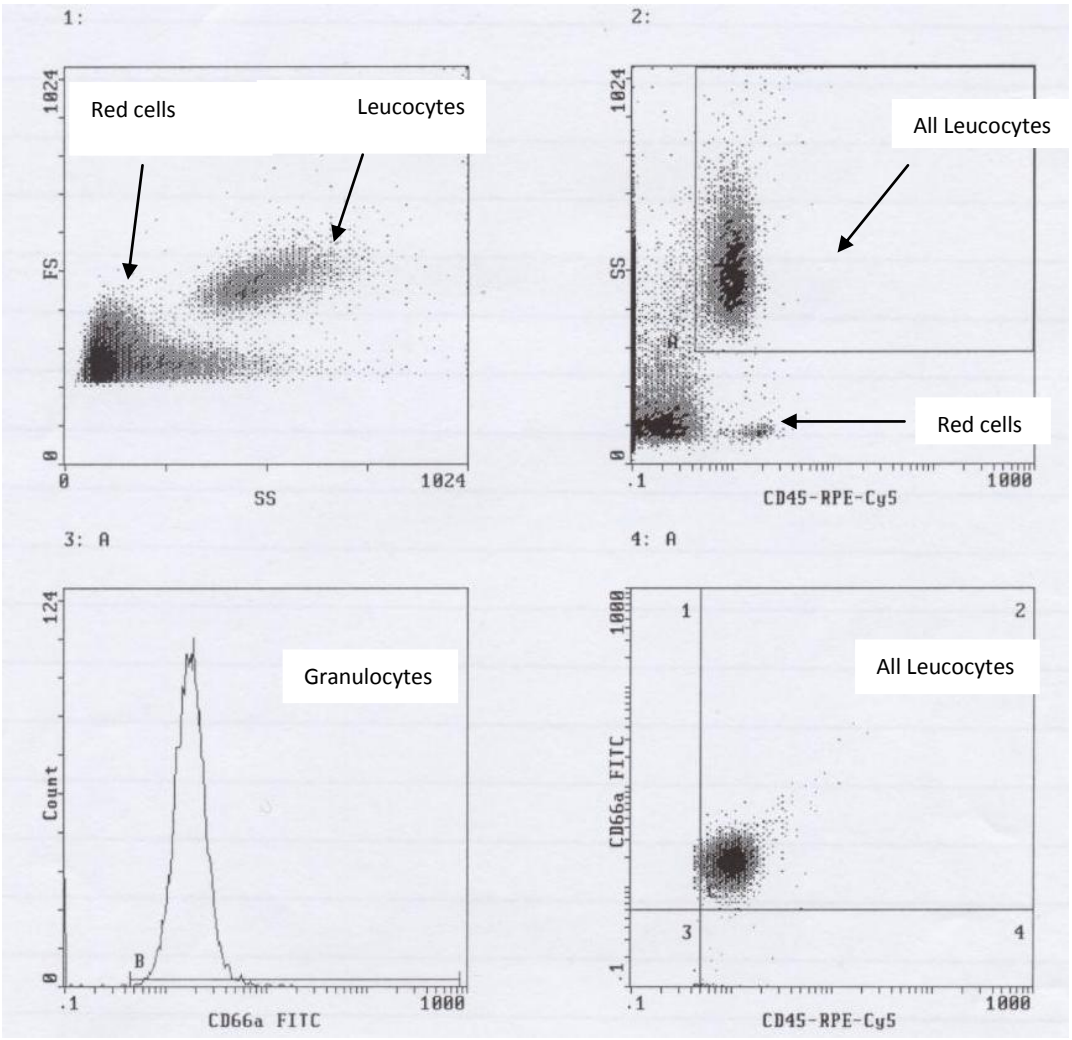
Thirty samples were also analysed in a cell counter, Coulter® A^C.T diff Analyzer, for granulocyte count. Even though this is not an accurate method for determining the cell count, it gives an idea of the proportion and the type of cells present. The granulocyte count of the PMN cells layer by FCM was 98.4%, 99.7% and 99.8% for 1 volunteer and 2 patient samples respectively. These values corresponded to 83.8%, 85% and 94% respectively in the cell counter. All 30 samples analysed had values ranging between 83.8% and 94% granulocytes.



Hist	Region	ID	%	Count
2	A	ALL LEUCOCYTES	28.1	9851
4	C1	C	2.13	210
	C2	C	96.0	9459
	C3	C	0.57	56
	C4	C	1.28	126

Hist	Region	ID	%	Count
3	B	GRANULOCYTES	98.4	9690

Figure 2-4. Flow cytometry result from one sample. Histogram 1 shows all cell types in the sample by side and forward scatter. Histogram 2, using CD45-RPE-Cy5 negative control, Gate A shows all leucocytes. Histogram 3, Gate B set for granulocytes using CD66a FITC, showing granulocyte count of 98.4%. Histogram 4 with both CD45-RPE-Cy5 and CD66a FITC identifying granulocytes and PBMC.

2.4 Polymerase Chain Reaction (RT-qPCR)

Figure 2-5 summarizes the processes involved in obtaining PCR product from whole blood.

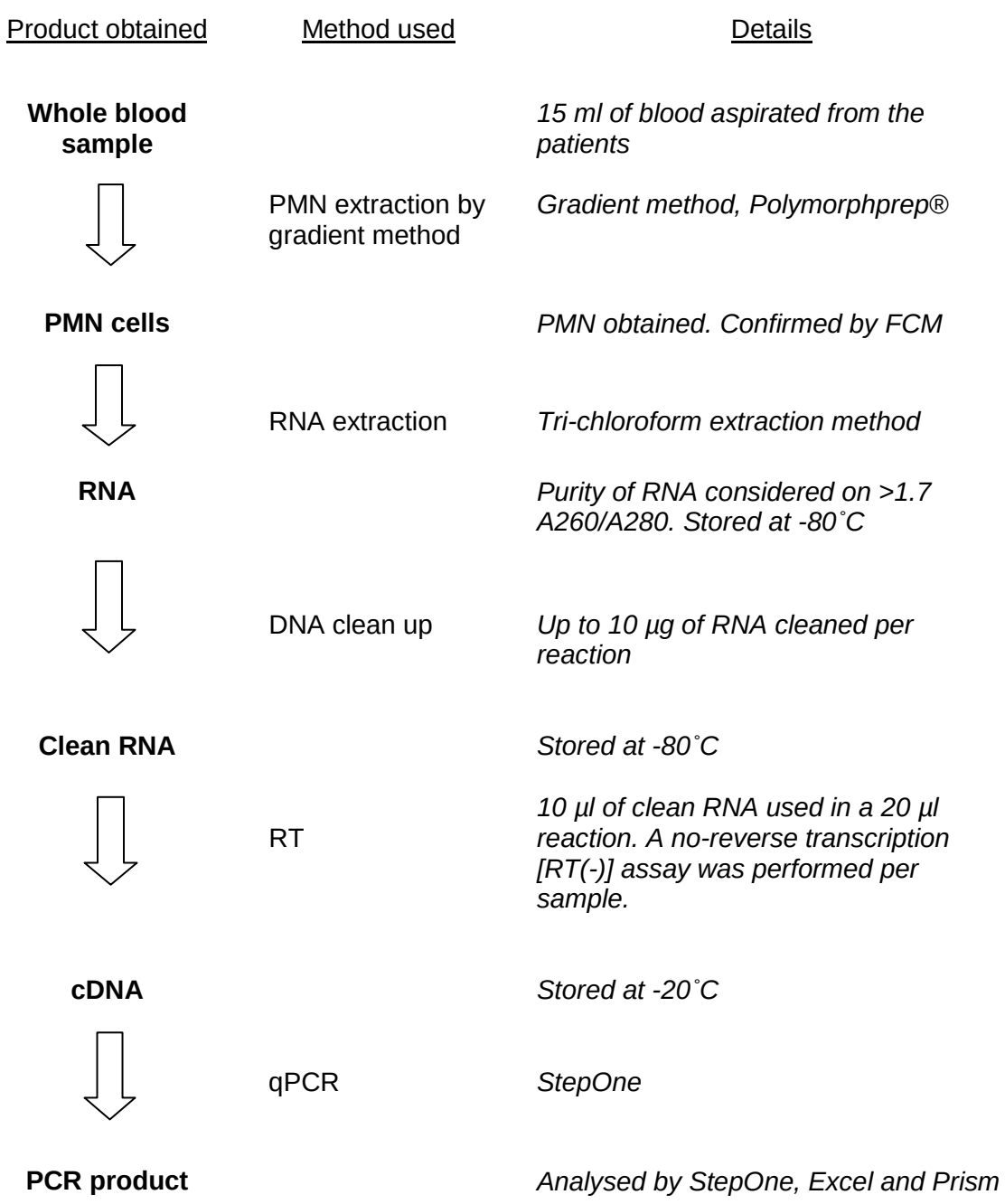


Figure 2-5. Flow chart of steps taken from whole blood sample to qPCR results.

2.4.1 Theory

To analyse the expression of NOP and ppN/OFQ genes in polymorphonuclear cells, we used Reverse Transcription - Real Time – Quantitative - Polymerase Chain Reaction (RT-qPCR).

The human genome is comprised of approximately 30,000 (named and unnamed) genes that represent the entire DNA sequence and is found in every cell of the human body.[105] A gene is a sequence of DNA which has nucleotides as its basic unit and has sets of exons (regions of DNA that encode for protein) and introns (regions of DNA that do not encode for protein). Each human cell transcribes different regions of DNA into mRNA in the nucleus and then in the cytoplasm the mRNA is translated into protein (**Figure 2-6**).

From DNA to protein

Double-stranded DNA is transcribed into single-stranded pre-mRNA (always synthesised from 5’ end to 3’ end) in the cell nucleus by RNA polymerase II. Pre-mRNA undergoes various modifications, including splicing, before being released into the cytoplasm. The non-coding regions (introns) are spliced out from the pre-mRNA to form the mature mRNA.

mRNA is then translated into protein by using transfer RNA (tRNA) and ribosomes. Further modification of proteins occurs to ensure their activation and stability. It is important to note that not all mRNA is translated into protein, which means that by using PCR we are able to identify the transcription of a given gene but cannot know if there is functional protein produced.

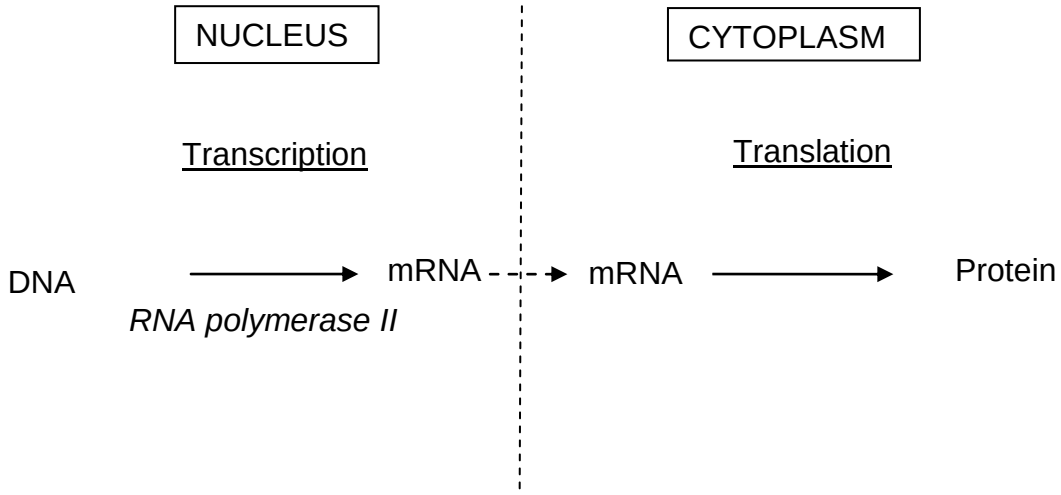


Figure 2-6. Schematic representation of conversion from DNA to Protein in the cell.

PCR

Polymerase chain reaction is a method of identifying the presence of a given gene by replicating its DNA sequence into millions of copies to make it detectable. A variety of samples can be analysed, blood, tissue, and cell cultures, amongst others. Very small amounts are sufficient to perform PCR. There are four basic components of a PCR reaction:

1. Two primers, forward and reverse. These are known short nucleotide sequences that synthesize the DNA in the 5’ to 3’ direction.

2. Thermo-stable DNA polymerase (an enzyme originally derived from the bacterium *Thermos aquaticus)* to synthesise the complementary DNA. This enzyme remains stable with high temperatures from PCR, in contrast to human DNA polymerase which is not stable at such high temperatures.

3. Free deoxynucleotides for the new complementary strand.

4. The sample containing a small amount of DNA with the sequence of interest.

During PCR the DNA sample is exposed to a temperature of 90˚C which denatures the double strand into 2 single strands (**Figure 2-7**). The primers then bind the single strands (annealing) and extend the sequence by using free deoxynucleotides and DNA polymerase, forming 2 DNA sequences. Annealing occurs at 40˚C and DNA synthesis at 70˚C. All this process, from denaturation of 1 DNA to synthesis of 2 DNA represents one PCR cycle. During the PCR cycles (usually 40 - 50) there is an exponential increase of the original amount of DNA at each cycle.

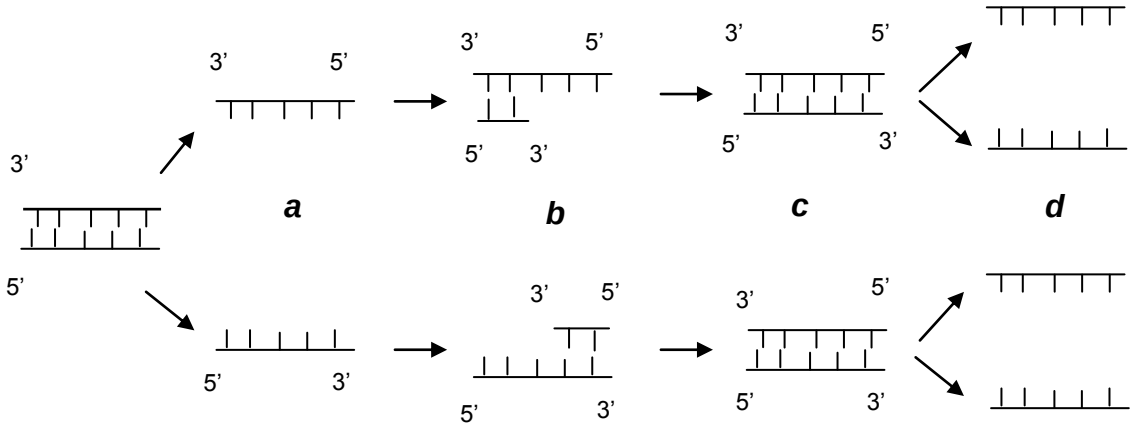


Figure 2-7. Schematic representation of one PCR cycle. **a** denature of double strand DNA sequence at 90˚C. **b** Annealing of forward and reverse primers at 40˚C. **c** Extension of cDNA by DNA or TAQ polymerase at 70˚C, duplicating the original amount of DNA. **d** New cycle starts with denaturing at 90˚C.

PCR can be performed from genomic DNA (containing introns and exons) or from mRNA (only exons), the latter called Reverse Transcription-PCR (RT-PCR). In RT-PCR mRNA is extracted from the tissue and reverse transcribed

to complementary DNA (cDNA) by means of the reverse transcriptase (**Figure 2-8**). cDNA is then replicated using the PCR technique as described above.

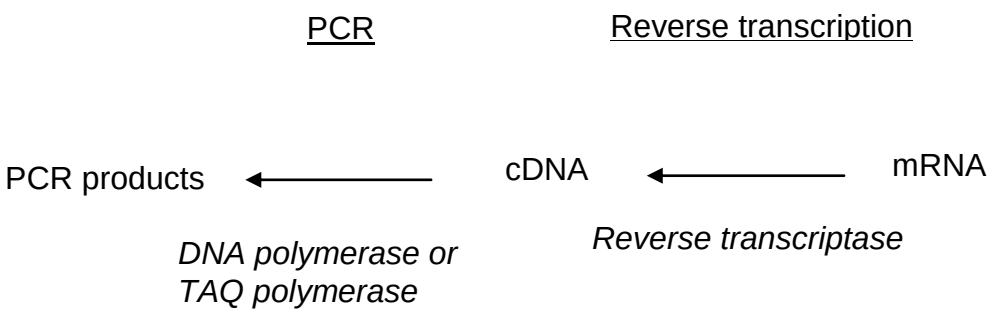


Figure 2-8. Schematic representation of reverse transcription and PCR. cDNA, complementary DNA.

Real time PCR can be performed by using fluorescence which allows for quantifying the amount of genetic material at each cycle. One of the most frequently used methods is based with TaqMan probes. These consist of a nucleotide sequence which has a fluorophore attached at the 5' end and another molecule, a quencher, at the 3'. The proximity of the quencher to the fluorophore prevents it from releasing fluorescence (Fluorescence resonance energy transfer, FRET) therefore it does not fluoresce whilst in solution. During the annealing phase the probe is attached to the DNA and the primer is extended with the Taq polymerase (a type of polymerase enzyme). The fluorophore is separated from the quencher when hydrolised by the Taq polymerase. The amount of fluorescence produced is directly proportional to the amount of PCR product (**Figure 2-9**).

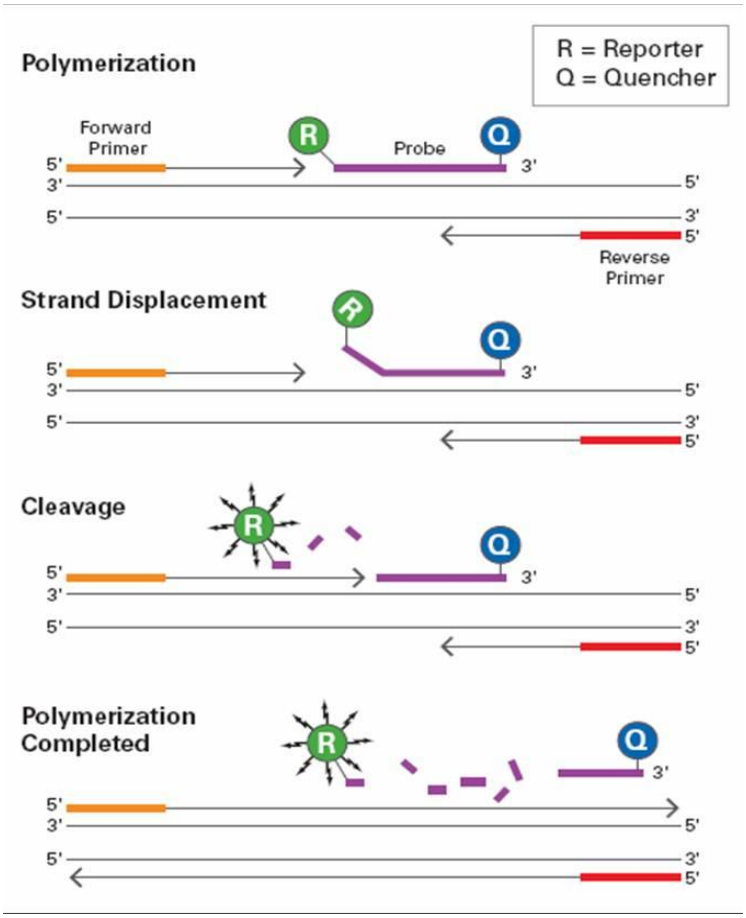


Figure 2-9. Schematic representation of PCR using TaqMan reagents. From Applied Biosystems StepOne™ Guide.[106]
http://www.appliedbiosystems.com/etc/medialib/appliedbio-media-library/images/application-and-technology/real-time-pcr/taqman-sybr-green-chemistry/data-images.Par.74690.Image.302.517.1.gif.Figure_2.gif

2.4.2 Materials

RNA extraction:

Tri – Chloroform method

99% Chloroform - *Sigma*,

Tri-reagent® *Sigma-Aldrich*,

Isopropanol, 75% ethanol, Tris-EDTA (TE) buffer.

*mirVana™ extraction method (*isolation kit, *Ambion*, by Applied Biosystems)

Chloroform - *Sigma*

100% Ethanol

miRNA Wash Solution 1

miRNA Wash Solution 2/3

Collection tubes and filter cartridges

DNA clean-up:

TURBO DNA free™ kit: Turbo DNase, 10x turbo DNase buffer, DNase inactivation reagent, nuclease-free water – *Ambion, UK*

Reverse transcription (RT):

High-Capacity Reverse Transcription Kit, *Applied Biosystems:* 10x RT buffer, 10x RT Random Primers, 25x dNTP Mix, MultiScribe™ Reverse Transcriptase, RNase Inhibitor

Quantitative Polymerase Chain Reaction (q-PCR):

2x TaqMan® Universal PCR MasterMix, Gene expression assay for GAPDH, gene expression assay for N/OFQ (Prepronociceptin, pNOC), Gene expression assay for NOP, and nuclease-free water. *Applied Biosystems*

Assay ID:

- For ppN/OFQ: ppNOC HS 00173823-m1
- For NOP: ORL-1 HS 00173471-m1
- For GAPDH: 4326317E

2.4.3 RNA extraction

Tri – Chloroform method

For the study in patients undergoing cardiac surgery under CPB, the Tri-chloroform extraction method[107] was used. PMN leucocytes were extracted from whole blood and stored in 1 ml of Tri-reagent® (deactivates RNases) at -80˚C, as described above (polymorphonuclear leucocytes extraction). Batch analysis was performed. The samples were defrosted and treated according to the following protocol:

1. Add 200 µl of chloroform
2. Mix by vortex for 2 minutes
3. Stand at room temperature for 3 minutes
4. Centrifuge at 16,000x g, room temperature, 15 minutes
5. Remove upper aqueous layer (containing mRNA) to a clean eppendorf tube

6. Add 500 µl of Tri-reagent® and 100 µl of chloroform
7. Mix by vortex for 2 minutes
8. Stand at room temperature for 3 minutes
9. Centrifuge at 16,000x g, room temperature, 15 minutes
10. Remove upper aqueous layer (containing mRNA) to a clean eppendorf tube
11. Add 500 µl of Isopropanol
12. Stand at room temperature for 10 minutes
13. Centrifuge at 16,000x g, room temperature, 10 minutes to obtain a pellet.
14. Discard the supernatant and resuspend the pellet in 75% ethanol.
15. Keep the pellet on ice for up to 1 h and then quantify.

For quantification, the alcohol was discarded and the pellet resuspended in up to 100 µl of Tris-EDTA (TE). 2 µl of this sample was added to 48 µl of TE; this was quantified using absorbance at 260 nm with the Eppendorf Biophotometer. Purity was assessed using the absorbance ratio between 260 nm and 280 nm and considered acceptable when > 1.7.

mirVana™ extraction method

For the sepsis study the mirVana™ isolation kit method was used. mirVana™ isolation kit, *Ambion*, by Applied Biosystems, is designed for purification of small interfering RNAs (siRNA) and microRNAs (miRNA). The mirVana kit was tested for use in PMN cells in our laboratory and we obtained high yields of

high quality mRNA in under 1 hour. We performed batch analysis following the protocol:

1. Add 200 µl of chloroform to the PMN cells (already on 1 ml of Tri-Reagent®)
2. Vortex for 30 – 60 seconds and centrifuge at 10,000x g at room temperature for 5 minutes
3. Recover the upper aqueous phase carefully to a fresh tube
4. Add 100% ethanol 1.25 volumes.
5. For each sample place a Filter Cartridge into a collection tube and pipette the ethanol/sample mixture onto the filter cartridge.
6. Centrifuge for 30 seconds at 10,000x g at room temperature to pass the mixture through the filter and discard the flow-through.
7. Use the same collection tube to collect the remainder of the sample and for the washing steps (up to 700 µl can be applied to a filter cartridge).
8. Apply 700 µl of 'Wash Solution 1' to the filter cartridge and centrifuge at 10,000x g at room temperature for 30 seconds, and discard the flow-through.
9. Apply 500 µl of 'Wash Solution 2/3' and centrifuge for 30 seconds, and discard the flow-through.
10. Repeat previous step, apply 500 µl of 'Wash Solution 2/3' and centrifuge for 30 seconds, and discard the flow-through.
11. Centrifuge for 1 minute to remove any residual fluid from the filter.
12. Transfer the filter cartridge to a fresh collection tube and add 100 µl

of pre-heated (95˚C) nuclease-free water.

13. Centrifuge at 10,000x g at room temperature for 30 seconds to recover the RNA.

2.4.4 RNA Quantification

For RNA quantification, 2 µl of the recovered sample were added to 48 µl of nuclease-free water; this was quantified using absorbance at 260 nm with the Eppendorf Biophotometer. Purity was considered with an absorbance ratio between 260 nm and 280 nm with a minimum of 1.7 taken as acceptable. RNA samples were stored at -80˚C.

2.4.5 Removal of DNA contamination

The RNA extraction method does not consistently yield RNA that is free from DNA contamination; therefore we used a kit to remove genomic DNA, TURBO DNA-*free*™ Kit, *Ambion*, prior to RT-PCR. Up to 10 µg of mRNA were cleaned from DNA contamination. For each sample, a 50 µl reaction was prepared containing 1 µl Turbo DNase, 5 µl 10x turbo DNase buffer, sample volume containing up to 10 µg mRNA and molecular biology grade water to a total volume of 50 µl. This was mixed and incubated at 37˚C for 30 minutes. Then 5 µl of DNase inactivation reagent was added, mixed by vortex, and left to stand for 3 minutes at room temperature mixing intermittently. The samples were then centrifuged at 10,000x g, at room temperature for 30 seconds to precipitate the inactivation reagent. The supernatant containing clean mRNA was transferred to a clean tube and stored at -80˚C.

2.4.6 Reverse Transcription (RT)

mRNA cleaned from DNA contamination was reverse transcribed to obtain complimentary DNA (cDNA). A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used. Reverse transcription was carried out in duplicate for RT (+) and single for RT (-) (control, without reverse transcriptase). A 20 µl reaction was prepared for each RT (+) and RT (-) for each sample. The following mix was used:

	RT (+)	RT (-)
10x Reverse transcription buffer	2 µl	2 µl
25xDeoxynucleotidetriphosphates (dNTP)	0.8 µl	0.8 µl
10x RT Random Primers	2 µl	2 µl
MultiScribe ™ Reverse Transcriptase	1 µl	0
RNase Inhibitor	1 µl	1 µl
Nuclease-free water	3.2 µl	4.2 µl
RNA sample	10 µl	10 µl
TOTAL	20 µl	20 µl

The mixtures were processed in an eppendorf Mastercycler™ according to the following protocol:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25˚C	37˚C	85˚C	4˚C
Time	10 min	120 min	5 min	Hold

The cDNA obtained was stored at -20˚C for batch qPCR analysis.

2.4.7 qPCR development

Quantitative real time PCR (qPCR) was performed using TaqMan probes and gene expression assays from Applied Biosystems and the StepOne™ Real-Time PCR instrument from Applied Biosystems.

Reactions were performed in duplex (both NOP and GAPDH or ppNOC (pre-pro N/OFQ) and GAPDH primers included in the same reaction). Reaction efficiencies for NOP had been determined previously by McDonald and colleagues[11] using serial dilutions of SH-SY5Y (a neuroblastoma expressing gene of interest) cDNA with GAPDH alone (singleplex) or with NOP (duplex). The efficiencies were calculated using the formula Efficiency=($10^{[-1/slope]}$-1x100). Efficiencies obtained were 94% and 98.9% for GAPDH, and 100% and 100% for NOP in singleplex and duplex respectively. The accepted range for efficiency is 90-110%.

Normalization was performed with GAPDH as a reference gene to control for variations in the various stages of mRNA extraction, reverse transcription and efficiencies of amplification. Ct values for GAPDH expression by PMN cells in septic patients and patients undergoing an inflammatory response were constant. The value of GAPDH as internal control has been corroborated by its stability in PMN cells in septic patients when analysed by the software GeNorm (unpublished data by our laboratory).

PCR set up

The following mixture was used per sample per gene of interest (NOP or ppN/OFQ):

2x TaqMan® Universal PCR Mastermix	10 µl
Assay mix for GAPDH	1 µl
Assay mix for gene of interest	1 µl
Nuclease-free water	6 µl
Sample	2 µl
TOTAL	20 µl

The thermal cycling conditions used during qPCR are shown in **Table 2-1**

PCR step	**Initial Steps**		**PCR (40 cycles)**	
	AmpErase® UNG Activation	AmpliTAQ Gold® DNA polymerase activation	Denature	Anneal/Extend
	HOLD	HOLD	CYCLE	
	2 min @ 50˚C	10 min @ 95˚C	15 sec @ 95˚C	1 min @ 60˚C

Table 2-1. PCR thermal cycling conditions. AmpErase® UNG, uracil-N-glycosylase, is used to prevent contamination from previous PCR products (carry-over) where dUTP has been used in the master mix. UNG cleaves the Uracil base from Uracil-containing DNA but does not affect natural DNA (thymine-containing DNA).[108]

Data analysis was performed using StepOne™ software version 2.0.

Ct or cycle threshold is the cycle at which the fluorescence reaches a predetermined threshold. We used Ct values to analyse PCR results. The ΔCt, and ΔΔCt are commonly used in PCR studies. ΔCt is the difference between Ct of the gene of interest (i.e. NOP or ppN/OFQ) and Ct of the reference gene GAPDH. **Figure 2-10**. The ΔΔCt refers to the difference of ΔCt in between two samples.

The maximum arbitrary cut-off point for reporting Ct values is 40. However, we observed that Ct values for ppN/OFQ were around 40, and these were higher after the inflammatory response. Therefore we accepted values above 40 (average ppN/OFQ Ct 34 to 42) when they were reproducible on repeated analysis. High Ct values indicate low mRNA expression of the gene of interest. We also use the $2^{-\Delta\Delta Ct}$ method, or fold change, to analyse PCR results.

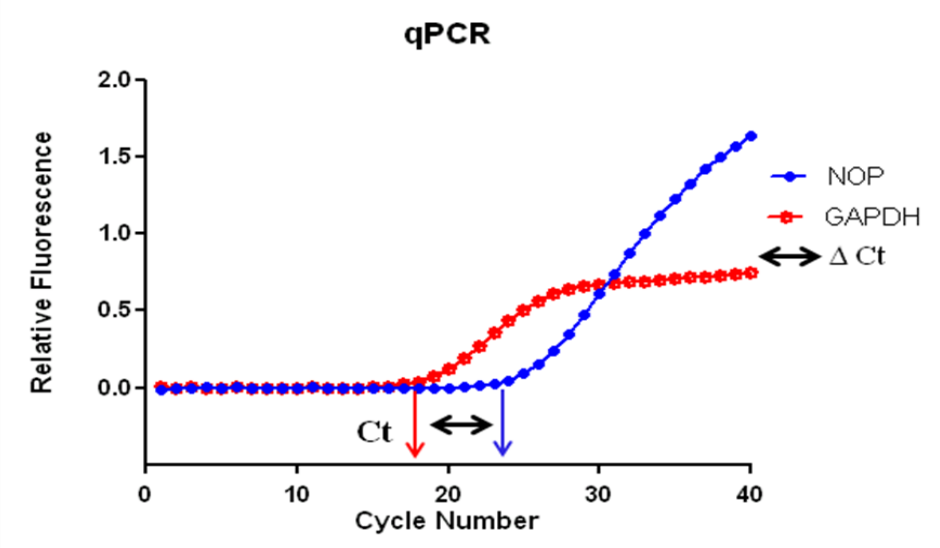


Figure 2-10 Graphical sample of qPCR result for NOP from one patient. Showing Ct for NOP= 24, where the fluorescence increases above the threshold line, Ct for GAPDH= 18, and therefore ΔCt= 6 (NOP Ct minus GAPDH Ct). The higher the Ct and ΔCt, the lower the gene expression. The values given are approximate for the purpose of explanation.

2.5 Radioimmunoassay

2.5.1 Theory

Radioimunoassay (RIA) is a technique used to measure concentrations of antigens by the use of antibodies. It was introduced in 1960 by Berson and Yalow.[109] To perform RIA it is necessary to have a radio labelled antigen (e.g. ^{125}I-N/OFQ), a specific antibody (e.g. specific for N/OFQ) and some method of separating the bound antibody from the free labelled peptide (i.e. secondary antibody).[109] A known amount of radio labelled antigen is mixed to the specific antibody to form a labelled antigen-antibody complex. The sample with the unknown concentration of peptide is added and this peptide competes with the labelled antigen (^{125}I-N/OFQ in this case) for binding sites at the specific primary antibody. As the assay is competitive, binding of ^{125}I-N/OFQ is low at higher peptide concentrations in the sample and vice versa. A secondary antibody is used to precipitate the antigen/antibody complexes thus allowing for measuring the radioactivity in the pellet with the means of a γ-counter (**Figure 2-11**). A standard curve is drawn and used to derive the unknown plasma concentrations.

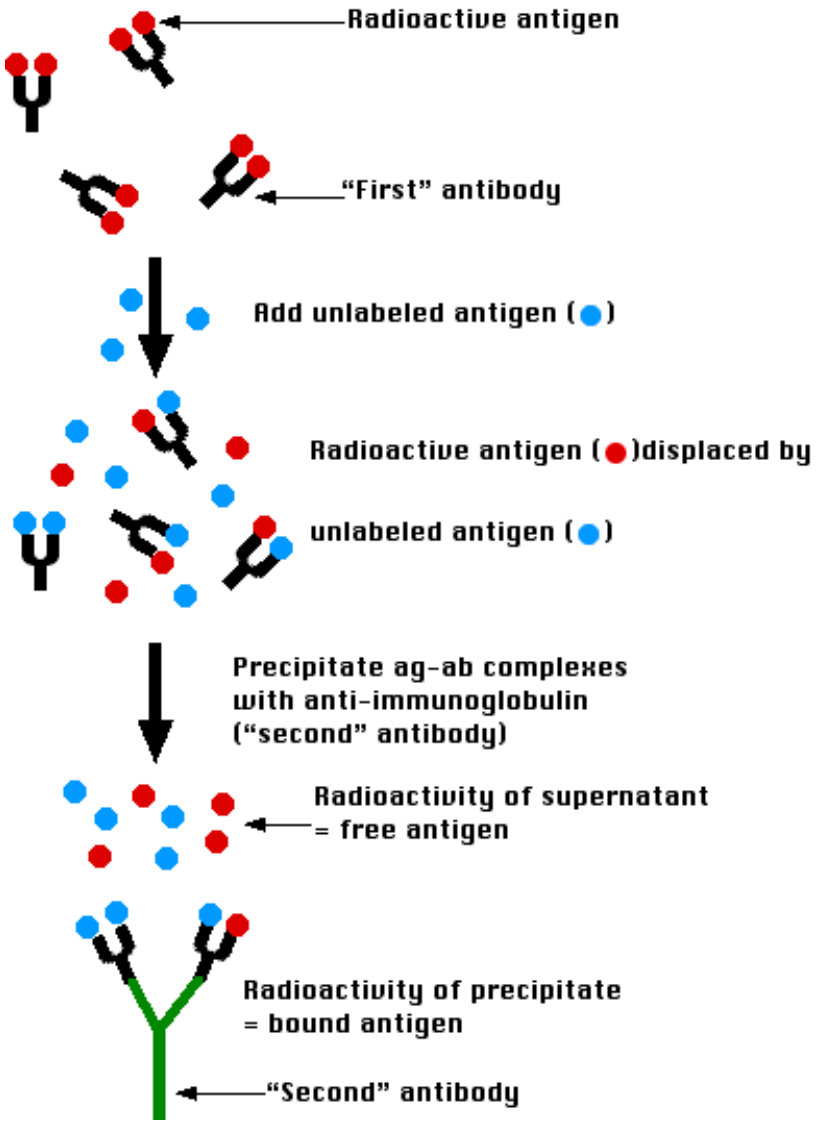


Figure 2-11. Schematic representation of radioimmunoassay. Taken from http://www.immunoassay.org/radioimmunoassay-ria/

2.5.2 Materials

Aprotinin 0.6 $TIU.ml^{-1}$ of blood

Acetonitrile

Trifluoroacetic acid (TFA)

Nociceptin.Opioid Receptor-Like (ORL_1) Peptide (Orphanin FQ) RIA kit – *Phoenix Pharmaceuticals, Inc*

2.5.3 Solid phase extraction

Plasma samples stored at -80°C were used for batch measurement of plasma N/OFQ concentrations. Blood sample collection was described above.

N/OFQ solid phase peptide extraction was performed using *Strata* cartridges containing 200mg of C18-E. The cartridges were prepared by washing with 1 ml of 60% acetonitrile in 1% TFA followed by 3 ml of 1% TFA 3 times. Two ml of plasma sample were acidified with equal volume of 1% trifluoroacetic acid (TFA) for 30 minutes at 4˚C and then loaded into the column. The columns were then washed with 3 ml of 1% TFA twice and the N/OFQ peptide then eluted by adding 2.5 ml of 60% acetonitrile in 1% TFA to the columns. The eluate samples were then dried at room temperature using a centrifugal evaporator connected to a vacuum pump and stored at -20˚C until batch assay.

2.5.4 Radioimmunoassay stage

A radioimmunoassay kit from *Phoenix pharmaceuticals* was used. The manufacturers' protocol was followed:

1. *Reconstitutions*:
    - Dilute the RIA buffer with 150 ml of distilled water – this is used to reconstitute all the other compounds and to dilute the samples
    - Reconstitute the standard peptide with 1 ml of RIA buffer. Mix well and store on ice.
    - Reconstitute the primary antibody (rabbit ant-peptide serum) with 13 ml of RIA buffer, mix well and store on ice

- Reconstitute the Positive Controls with 1 ml of RIA buffer. Mix well and store on ice.
- Reconstitute samples with RIA buffer
- Prepare dilutions of the standards as below:

Tube	Sample	RIA buffer	Standard concentration
Stock	Powder	1 ml	12.8 µg.ml^{-1}
0	10 µl of stock	990 µl	128,000 pg.ml^{-1}
A	10 µl of 0	990 µl	1,280 pg.ml^{-1}
B	500 µl of A	500 µl	640 pg.ml^{-1}
C	500 µl of B	500 µl	320 pg.ml^{-1}
D	500 µl of C	500 µl	160 pg.ml^{-1}
E	500 µl of D	500 µl	80 pg.ml^{-1}
F	500 µl of E	500 µl	40 pg.ml^{-1}
G	500 µl of F	500 µl	20 pg.ml^{-1}
H	500 µl of G	500 µl	10 pg.ml^{-1}

Table 2-2. Radioimmunoassay Standard dilutions

2. *Set up of initial RIA reactions:*
   - Add 200 µl of RIA buffer into each Non-specific Binding (NSB) tube
   - Add 100 µl of RIA buffer into each Total Binding (TB) tube
   - Add 100 µl of standards A through H into standard tubes
   - Add 100 µl of positive control (PC) into PC tubes
   - Add 100 µl of sample into sample tubes

- Add 100 µl of primary antibody into sample, standard and TB tubes
- Vortex, cover and incubate all tubes for 16 – 24 h at 4˚C

3. *Addition of radio labelled ^{125}I-N/OFQ antigen:*
   - Reconstitute the ^{125}I-N/OFQ with 13 ml of RIA buffer and mix to make tracer solution. Check the concentration of the tracer solution and adjust with RIA buffer to obtain a total activity of between 8,000 and 10,000 cpm/100 µl
   - Add 100 µl of ^{125}I-N/OFQ to each tube
   - Vortex and incubate all tubes for 16 – 24 h at 4˚C

4. *Addition of secondary antibody and γ-count*
   - Reconstitute the secondary antibody (Goat Anti-Rabbit IgG serum, GAR) with 13 ml of RIA buffer
   - Reconstitute the Normal Rabbit Serum (NRS) with 13 ml of RIA buffer
   - Add 100 µl of GAR to each tube, except TC tube
   - Vortex and incubate for 90 minutes
   - Add 500 µl of RIA buffer to each tube except TC tube and vortex.
   - Centrifuge all tubes (except TC tube) at 1,700 x g for 20 minutes at 4 ˚C.
   - Remove supernatant and assay pellet for ^{125}I using a γ-counter (Packard-Cobra)

The final contents of each tube for the measurement of plasma N/OFQ peptide by RIA and the standard curve obtained are shown in **Table 2-3** and **Figure 2-12** respectively.

Sample tube	RIA buffer	Standard	Sample	Primary antibody	^{125}I-N/OFQ	Secondary antibody, GAR	NRS
TC					100		
NSB	200				100	100	100
TB	100			100	100	100	100
Standard		100		100	100	100	100
PC			100	100	100	100	100
Sample			100	100	100	100	100

Table 2-3 Summary of content in each RIA tube. Values expressed in µl. TC, total count; NSB, non-specific binding; TB, total binding; PC, positive control (supplied by manufacturer).

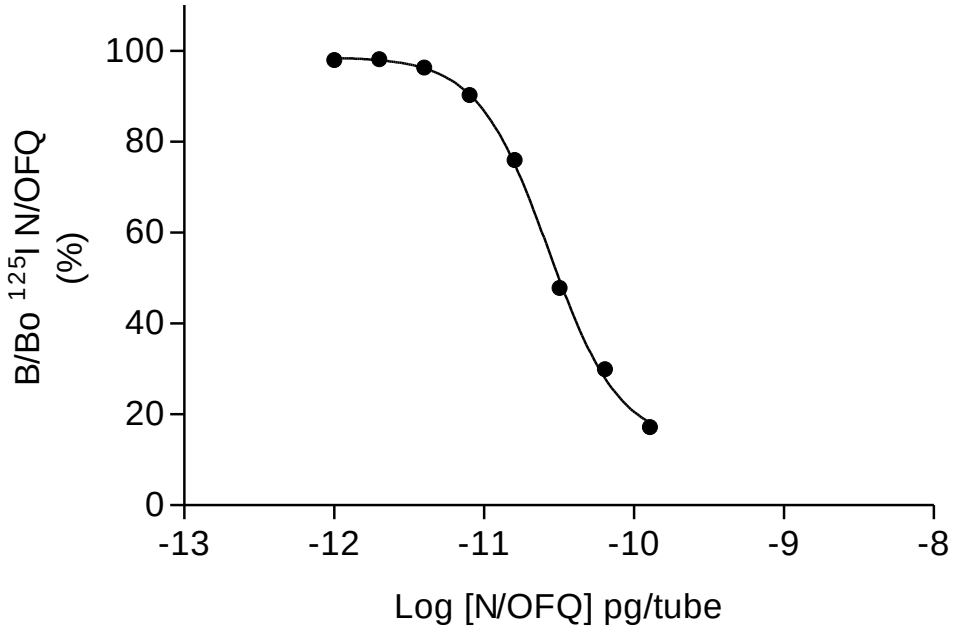


Figure 2-12. Radioimmunoassay standard curve. Proportion of Bound to unbound (B/Bo) ^{125}I-N/OFQ as a function of added N/OFQ standard. The higher the standard (or sample) N/OFQ peptide concentration the lower the radio labelled ^{125}I-N/OFQ bound. The standard curve enables for the unknown concentrations in plasma to be estimated.

Assay quality control data

In this assay for the cardiac study samples, 9327 cpm (counts per minute) were added to each tube and 38.2% were bound in the total assay tubes (product reference sheet Lot 506508 indicates 37%). The IC_{50} for the standard curve was 27 $pg.ml^{-1}$ (product reference sheet Lot 506508 quotes 15.47 $pg.ml^{-1}$). Quadruplicate analysis of the positive control gave an intra-assay CV of 6.27%.

2.6 ELISA

2.6.1 Theory

Various plasma inflammatory markers were analysed in both cardiac and septic studies. Cardiopulmonary bypass (CPB) is known to cause increased plasma concentrations of IL-6, IL-8, IL-10, IL-18 and TNFα.[99;100] IL-8 has been associated with new onset atrial fibrillation post-operatively,[110] IL-10 associated with development of organ failure[111] and TNFα with more complicated recovery based on inotrope requirement and plasma lactate.[112] Myeloperoxidase (MPO) is an enzyme released by activated neutrophils and is an indicator of inflammation and sepsis.[91]

We investigated the plasma concentrations of IL-8, IL-10, TNFα and MPO by Enzyme-linked immunosorbent assay (ELISA). These are known inflammatory markers that will allow us to correlate the patient's inflammatory response with changes in plasma N/OFQ concentrations and in mRNA expression of the N/OFQ system.

The principles of ELISA are similar to those of RIA. A capture antibody is used to coat the wells where the sample with an unknown amount of antigen or the standard is added, forming an antigen-antibody complex, (**Figure 2-13**). A detection antibody that is linked to an enzyme then binds to the antigen-antibody complex. In the final step a chemiluminescent reagent, such as streptavidin-HRP, is added and the enzyme linked to the detection antibody converts the streptavidin-HRP into a detectable signal which is then read by a microplate reader. The amount of signal produced is directly proportional to the amount of unknown antigen.

A standard curve is created by plotting t each standard concentration against the absorbance and drawing a best fit curve, the sample concentrations are then derived from the standard curve (we used GraphPad Prism version 5.0), It is recommended to include a standard curve for each ELISA plate. There are known variations in the ELISA results caused by different factors. A coefficient of variation (CV) can be calculate to indicates any inconsistencies and inaccuracies in the results. The coefficient of variation is the ratio of the standard deviation to the mean (CV = standard deviation / mean). It is expressed as a percentage of variance to the mean. Fluctuations in the CV can be attributed to inaccurate pipetting, bacterial contamination of samples or reagents, splashing of reagents between wells, cross contamination between reagents, temperature variations across the plate, and wells drying out if there are draughts or are not covered at incubation steps.[113]

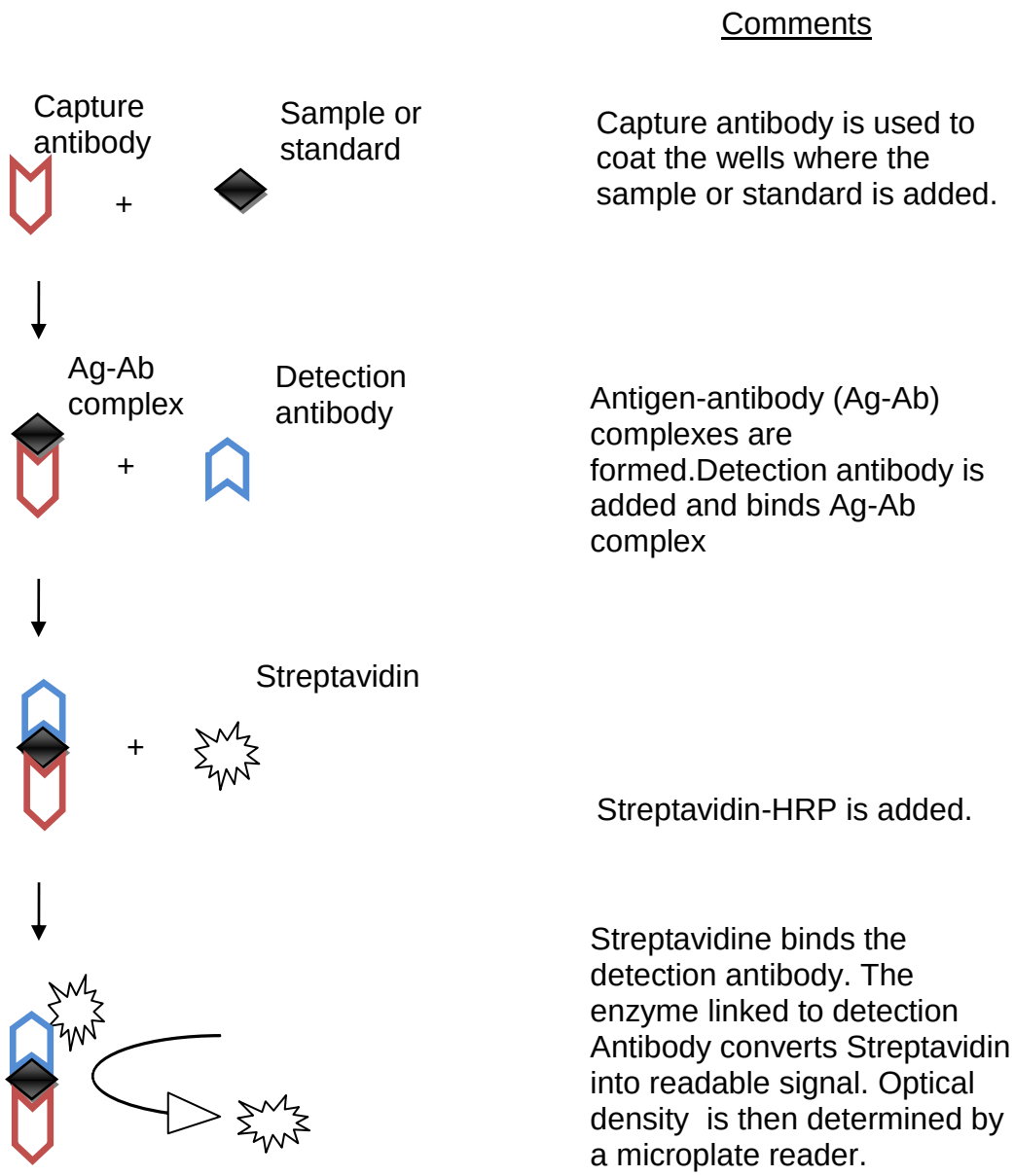


Figure 2-13. Schematic representation of basic principles of ELISA. Adapted from http://www.rndsystems.com/product_detail_objectname_mosaic_assay_principle.aspx Accessed 29/02/2012.

2.6.2 Materials

- DuoSet® ELISA Development Kit for IL-8, IL-10 and TNFα - *R&D systems*
- Phosphate Buffered Saline, PBS.
- Foetal Bovine Serum, FBS.
- Wash Buffer: 0.05% tween® 20 in PBS.
- Reagent Diluent: 1% Bovine serum albumin (BSA) in PBS.
- ILMA (Immunoluminometric assay buffer) 1 litre containing: Sodium phosphate 1.5 $mmol.l^{-1}$, disodium hydrogen phosphate 8 $mmol.l^{-1}$, sodium chloride 140 $mmol.l^{-1}$, EDTA 1 $mmol.l^{-1}$, BSA 1 $g.l^{-1}$, sodium azide 0.1 $g.l^{-1}$, Triton x 100 0.1% 1 ml. pH 7.04
- Buffer B wash 10 litres containing: Sodium chloride 197.5 g, sodium phosphate 2.3 g, disodium hydrogen phosphate 11.3 g, sodium azide 1 g, Tween®20 5 ml.
- Streptavidin-HRP.

2.6.3 ELISA method

Inflammatory markers were measured in plasma obtained from whole blood as described under 'Blood collection' above. Batch analysis was performed using an ELISA kit from *R&D systems*. The manufacturer's protocol was followed:

1. *Reconstitution of reagents*
   - Capture antibody: Add 1 ml of PBS to obtain a concentration of 720 $\mu g.ml^{-1}$ of mouse anti-human TNFα. Dilute with PBS to a working

concentration of 4µg.ml^{-1} (working concentrations for IL-8 and IL-10 were 4µg.ml^{-1} and 2µg.ml^{-1} respectively).

- Detection antibody: Add 1 ml of Reagent Diluent to obtain a concentration of 63 µg.ml^{-1} of goat anti-human TNFα. Dilute in ILMA to a working concentration of 350 ng.ml^{-1}.
- Standard: Add 0.5 ml of Reagent Diluent to obtain a concentration of 320 ng.ml^{-1} of recombinant human TNFα. Make 2-fold serial dilutions for a 6 point standard curve up to 1,000 pg.ml^{-1}.
- Streptavidin-HRP: Dilute to working concentration as specified on the vial.

2. *Plate preparation*
   - Coat plates with 100 µl of capture antibody, seal and incubate overnight at room temperature
   - Wash plates with PBS
   - Block plates by adding 200 µl of 10% FBS in PBS and incubate at room temperature for 2 h.
   - Wash plates with Buffer B

3. *Assay procedure*
   - Add 100 µl of ILMA buffer to each well
   - Add 100 µl of sample or standard, cover and incubate at room temperature for 2 h.
   - Wash plate with Buffer B

- Add 100 µl of Detection Antibody, cover and incubate at room temperature for 2 h.
- Wash plate in Buffer B
- Add 100 µl of Streptavidin to each well, cover and incubate at room temperature for 2 h.
- Determine the optical density of each well using a microplate reader.
- Values were derived from a standard curve (**Figure 2-14**), where log of relative light units (RLU) were directly proportional to log of plasma concentrations of the inflammatory marker (TNF-α in this example).

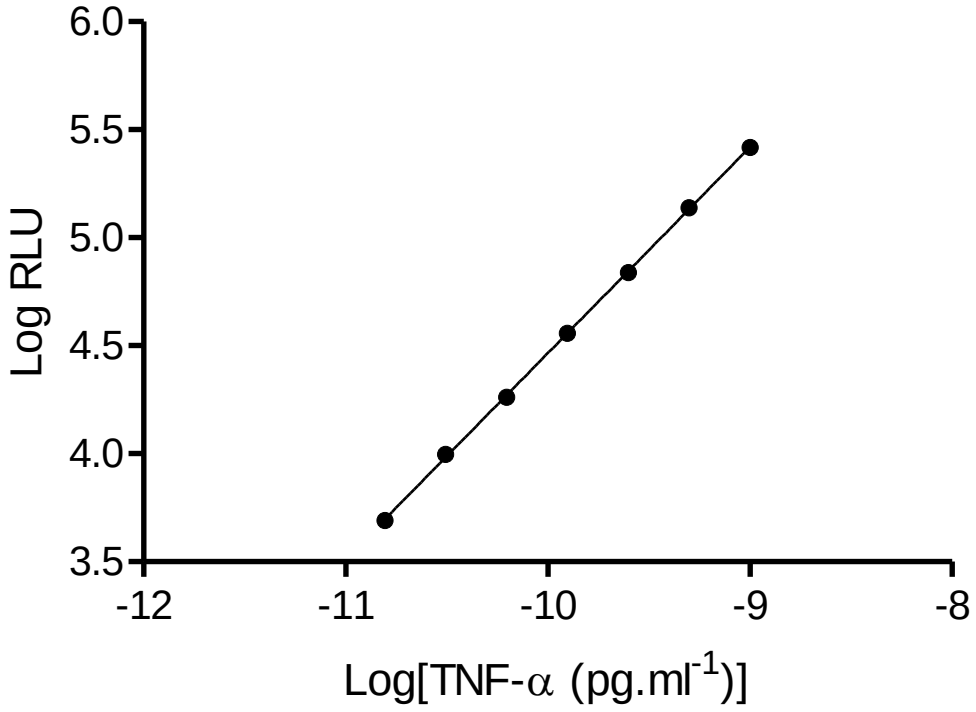


Figure 2-14 Example of a standard curve for cytokines using ELISA. A standard curve was included on each plate assayed.

2.7 Data collection and analysis software

The following computer software programmes were used for data collection and analysis:

StepOne™ software V 2.0, *Applied Biosystems* for PCR data

Microsoft office Excel 2007

GraphPad Prism version 5.0 for statistical analysis and graphical representation.

2.7.1 Data analysis

Statistical analysis was performed using GraphPad Prism. Analysis of data distribution was performed using D'Agostino & Pearson Omnibus normality test.

Numerical non-parametric distributed data (e.g. NOP and ppN/OFQ mRNA expression in the cardiac study) were analysed using Kruskal-Wallis analysis of variance (ANOVA) with Dunn's multiple comparison test for group analysis.
In the sepsis study the data from the recovery subgroup of 22 patients was assumed to be of non-parametric distribution due to the small sample size. Kruskal-Wallis ANOVA was also used in this case.

Parametric data (e.g. NOP and ppN/OFQ mRNA expression on D1 and D2 of sepsis) were analysed with student's t test for paired data.

Spearman's Correlation coefficient for non-parametric continuous data was used to see if there was a correlation between laboratory data (PCR results, N/OFQ concentrations and inflammatory markers) and physiological data (e.g. aortic cross clamp time, cardio-pulmonary bypass time, inotropic support, white cell count, and lactate concentrations).

3 The effect of Cardiac surgery under cardiopulmonary bypass on the N/OFQ system

3.1 Introduction

Cardiopulmonary bypass (CPB) is known to cause a Systemic Inflammatory Response Syndrome (SIRS)[99;100] due to mechanisms such as contact of blood with foreign surfaces in the extracorporeal circuit, non-physiologic pulsatile perfusion and ischemia-reperfusion injury due to cardioplegic arrest.[99] These mechanisms lead to:

- Activation of complement, kinin, fibrinolytic and coagulation cascades
- Endothelial cell activation
- Production of cytokines and of adhesion molecules
- Activation and aggregation of neutrophils and cellular immune system[100]

CPB is associated with increased plasma concentrations of the cytokines IL-6, IL-8, IL-10, IL-18 and TNFα.[99;100;114] **Table 3-1** shows previous studies on interleukins and CPB. Increased plasma concentrations of IL-8 have been associated with new onset atrial fibrillation post-operatively,[110] Increased IL-10 concentrations have been related to development of organ failure,[111] and increased TNFα concentrations are linked with a more complicated recovery after cardiac surgery (based on inotrope requirement and plasma lactate).[112]

CPB activates PMN cells by contact with the surface of the bypass circuit;[71] this PMN cell activation is then promoted by raised cytokines. Activated PMN cells

release proteolytic enzymes including myeloperoxidase (MPO, see Chapter 1: Introduction, inflammatory markers),[88] matrix-metalloproteinases (MMPs), PMN elastases and oxygen-free radicals,[89] which promote organ dysfunction.

All the above mechanisms can lead to derangement in coagulation pathways[98], altered regional organ perfusion, alterations in acid-base balance, intense systemic inflammatory activation, lung dysfunction,[89] renal impairment, gastrointestinal and metabolic changes, microemboli and neurological impairement.[115]

Limited data is available regarding the N/OFQ system in patients with SIRS and in patients with cardiac disease. Our research group has previously reported the presence of NOP mRNA in human right atrium (n=38), but not of ppN/OFQ mRNA (n=10), in patients with coronary artery disease undergoing cardiac surgery with CPB.[11] Fontana and colleagues[116] showed increased plasma concentrations of N/OFQ in patients with unstable angina (n=41) compared to those with stable angina (n=7) and controls (n=20), without changes in blood pressure or heart rate. On the other hand, Krepuska et al[6] showed lower plasma N/OFQ concentrations in patients with severe chronic angina (n=12) compared to patients with mild angina (n=10) and healthy controls (n=14). Stamer and colleagues[34] recently reported reduced ppN/OFQ mRNA expression in peripheral blood cells in 18 critically ill patients with sepsis (i.e. with SIRS and documented infection), and increased NOP mRNA expression in non-survivors of sepsis, compared with healthy controls. Williams and colleagues[33] also studied critically ill ICU patients with sepsis (n=21) and found

increased plasma N/OFQ concentrations in those who died compared to survivors.

It is unclear what the human response of ppN/OFQ and NOP to inflammation is, and whether the response of the N/OFQ system has an effect on patient outcome. Based on animal studies and the limited evidence available in humans (described in Chapter 1: Introduction), we hypothesise that plasma concentrations of N/OFQ are increased in patients undergoing CPB and that the origin of N/OFQ is the PMN cell, therefore we further hypothesise that mRNA expression for NOP and ppN/OFQ by the PMN cells is up-regulated. With this study we aimed to analyse the response of the N/OFQ system in plasma and in PMN cells, and its association with clinical outcome in patients undergoing cardiac surgery with cardiopulmonary bypass, as a model of the inflammatory response.

Cytokines studied	Methods	Results	Reference
IL-2, -6, -8 and -10, MCP-1, VEGF	60 patients undergoing CABG Group 1: Off pump Group 2: Pulsatile CPB Group 3: Linear CPB	↑MCP-1, VEGF, IL-6, -8and -10 in CPB ↓IL-2 in CPB	Onorati, 2010[99]
IL-1, -1ra, -6, -10 and TNFα	43 patients undergoing CABG with CPB	↑ IL-1, -1ra, -6, -10 and TNFα	Poulsen, 2009[117]
IL-6, -8 and -10	113 patients undergoing CABG with CPB	↑IL-6, -8 and -10 IL8 was related to new onset AF	Wu, 2008[110]
TNFα and IL-10 mRNA in PBMCs	82 patients undergoing cardiac surgery with CPB	↑TNFα and IL-10	Duggan, 2006[112]
IL-10	150 patients undergoing CABG with CPB	↑IL-10. IL-10 higher in patients with organ dysfunction	Galley, 2003[111]
IL-8, -10, -1ra and TNFα	20 patients undergoing cardiac surgery with CPB	↑IL-8, -10, and -1ra No change in TNFα	McBride, 1995[118]

Table 3-1 Evidence of increased inflammatory markers during cardiopulmonary bypass. MCP-1, Monocyte chemo-attractant protein; VEGF, vascular endothelial growth factor; CABG, coronary artery bypass grafting; CPB, Cardiopulmonary bypass;

3.2 Materials and Methods

3.2.1 Patients recruitment and sample collection

With local research ethics committee approval (LREC ref: 08/H0406/103) and written informed consent 40 patients undergoing cardiac surgery with Cardio-Pulmonary Bypass (CPB) at Glenfield Hospital, Leicester, were recruited between July 2008 and January 2010. Patients were ASA III and IV and aged over 18. Most patients had an arterial line placed before induction of anaesthesia. General anaesthesia was administered by the cardiac anaesthetist responsible for clinical care. Overall, all patients received the following drugs at induction of anaesthesia: alfentanil 5 – 10mg or fentanyl 0.5 – 1mg, and propofol or etomidate until loss of eyelash reflex. Vecuronium or rocuronium was given to produce neuromuscular blockade and facilitate tracheal intubation. Isoflurane 1 – 2% in an oxygen/air mixture was used for maintenance of anaesthesia. Morphine 10mg was administered for analgesia at the end of surgery. All patients had a central line and a pulmonary artery flotation catheter sheath inserted after induction of anaesthesia. Most blood samples were taken from the arterial line (in 2 patients the third blood sample was taken from the central line as the arterial line had been removed).
Blood samples, 22.5 ml at each sampling, were taken at the following times:

- Sample 1 (t0): Before induction of anaesthesia. 3 patients had t0 sample taken post-induction of anaesthesia as there was no arterial line present. We have previously seen that there is no difference in plasma N/OFQ concentrations comparing before and immediately after induction of anaesthesia [Median (IQR) = 5.8(1.2 – 10.8) and 3.4 (1.2 – 9) $pg.ml^{-1}$

respectively. P value = 0.8, data from 16 patients undergoing abdominal aortic aneurysm repair].[119] Based on these results, we did not consider necessary to standardise the anaesthetic technique.

- Sample 2 (t3): 3 hours post-start of CPB
- Sample 3 (t24): 18 to 24 hours post-start of CPB

3.2.2 Cardio Pulmonary Bypass pump

CPB pumps were primed following the standard local protocol at Glenfield Hospital with the following solutions:

Plasmalyte 148	1,000 ml
Colloid	500 ml
Mannitol 10%	200 ml
Sodium bicarbonate 8.4%	45 ml
Heparin	10,000 iu

3.2.3 Blood collection for plasma extraction

As described in Chapter 2: Methods, 7.5 ml of blood was aspirated from the arterial line into an EDTA bottle. 150 µl of aprotinin (containing 4.5 TIU, 0.6 $TIU.ml^{-1}$) were immediately added to prevent protein degradation. The samples were then transferred on ice to the laboratory and centrifuged at 3,000x g, 4˚C for 15 minutes. The plasma obtained was stored in 1ml aliquots at -80˚C and used for batch analysis of N/OFQ peptide by radioimmunoassay and of cytokines and MPO by ELISA.

3.2.4 Radioimmunoassay

Plasma N/OFQ concentration was measured in all 120 samples (3 samples per patient) by Radioimmunoassay. Details are described in Chapter 2: Methods.

3.2.5 ELISA

Known inflammatory markers were analysed by ELISA in all 120 samples. Plasma IL-8, IL-10, TNFα, and MPO concentrations were determined. Details are described in Chapter 2: Methods.

3.2.6 RT-PCR

Polymorphonuclear leucocytes were isolated from the blood samples and RT-PCR for NOP and ppN/OFQ mRNA expression performed. Details of polymorphonuclear extraction and RT-PCR are described in Chapter 2: Methods.

3.2.7 Statistical analysis

Physiological data, RT-PCR results, N/OFQ plasma concentrations and interleukin plasma concentrations were analysed for normality distribution using D'Agostino and Pearson Omnibus using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Statistical analyses used the non-parametric test, Kruskal-Wallis Analysis of Variance (ANOVA), as there were more than 2 groups (t0, t3 and Rec) and data were unpaired (some measured values were undetermined or below detection limit, therefore there was a need to use an unpaired test).

Spearman's Correlation coefficient for non-parametric data was used for analysis of the relationship between laboratory data (i.e. PCR results, N/OFQ concentrations, inflammatory markers) and physiological data, to determine the degree by which two measured variables were related.

Statistical significance was considered with p values <0.05.

3.3 Results

3.3.1 Baseline patient characteristics

Patient characteristics, previous medical and drug history, functional capacity and LV function are shown in **Table 3-2** .

Age (years)	70.5 (61.7 – 76)
Male/Female (n)	28/12
BMI ($Kg.m^{-2}$)	28 (25 – 33)
ASA physical status classification (n (%))	
III	33 (82)
IV	7 (17)
Co-morbidities (n (%)):	
Previous MI	5 (12)
Hypertension	28 (70)
Hypercholesterolemia	30 (75)
Diabetes	10 (25)
Smoker	5 (12)
COPD/Asthma	4 (10)
PVD	1 (2)
CVA	4 (10)
eGFR <90	28 (70)
Hypothyroid	3 (7)
Osteoporosis	1 (2)
Atrial fibrillation	7 (17)
Angina classification CCS (n (%))	
No angina	13 (32)
1	6 (15)
2	16 (40)
3	3 (7)
4	2 (5)
NYHA functional class (n, (%))	
1	19 (47)
2	14 (35)
3	7 (17)

Drug history (n (%)):	
ACEI/ARB	23 (57)
Diuretic	13 (32)
β blocker	25 (62)
α blocker	2 (5)
Ca^{2+} channel blocker	6 (15)
Anti platelet therapy	27 (67)
Aspirin	20 (50)
Clopidogrel	3 (7)
Aspirin+Clopidogrel	2 (5)
Aspirin+Dypiridamol	2 (5)
Nitrates	12 (30)
Nicorandil	5 (12)
Statins	30 (75)
Simvastatin	24 (60)
Atorvastatin	6 (15)
Anti-arrhythmics	4 (10)
Amiodarone	1 (2)
Digoxin	3 (7)
Insulin	3 (7)
Oral hypoglycaemic	5 (12)
Warfarin	4 (10)
LV function (n (%)):	
Good (EF > 50%)	25 (62)
Moderate (EF 30 – 50%)	12 (30)
Poor (EF <30%)	3 (7)

Table 3-2 Baseline patient characteristics. Values expressed as median (Interquartile range, IQR) or number (%). BMI, Body Mass Index; ASA, American Society of Anesthesiologists; CCS, Canadian Cardiovascular Society; NYHA, New York heart Association.

3.3.2 Intra-operative and postoperative data, and physiological variables

Surgical details, physiological variables and laboratory blood results at induction (t0), 3h (t3) and 18 – 24h (t24) post-CPB are shown in **Table 3-3** and **Table 3-4**. There was no significant difference between heart rate, mean arterial pressure, white cell count and neutrophil count at t3 compared to t0.

Surgical procedure (n (%)):		
CABG	17 (42.5)	
Valve replacement	15 (37.5)	
CABG + Valve replacement	7 (17.5)	
Aortic root replacement	1 (2.5)	
CPB time (mins)	89 (76 – 123)	
Aorta cross-clamp time (mins)	54 (45 – 74)	
Temperature on CPB (˚C)	32 (30 – 32)	
Postoperative pacing (n (%))	15 (37)	
Blood transfusion at t3 (n (%))	12 (30)	
Blood transfusion at t24 (n (%))	19 (47)	
Inotropic support (n (%))	t3	t24
Minimal (Dopamine ≤5mcg.kg^{-1}.min^{-1})	10 (25)	7 (17)
Moderate (Dopamine >5mcg.kg^{-1}.min^{-1} and/or adrenaline/noradrenaline ≤0.1mcg.kg^{-1}.min^{-1})	20 (50)	9 (22)
Maximal (Dopamine >15mcg.kg^{-1}.min^{-1}, and/or adrenaline/noradrenaline >0.1 mcg.kg^{-1}.min^{-1})	2 (5)	3 (7)
Intra-aortic balloon pump at t24 (n (%))	4 (10)	
ITU stay (days)	1 (1 – 3.7)	
Hospital stay (days)	15 (10 – 31)	
30 Day mortality (n (%))	1 (2)	

Table 3-3. Surgical details. Values expressed as median (IQR) or number (%).

	t0	t3	t24
HR (bpm)	64 (56 - 72)	85 (79 - 90)	84 (71 - 88)
MAP(mmHg)	90 (79 - 102)	70 (63 - 82)	75 (69 - 81)
Temperature (˚C)		36 (35.3 - 36.5)	36.8 (36.5 - 37.2)
SOFA score		5 (3.2 - 6)	4 (1 - 6)
WCC ($x10^9$/L)	7.9 (6.4 - 10)	10.5 (8.8 - 14.1)	10 (7.9 - 12.5)
Neutrophils (%)	66.5 (61 - 81)	83 (80.2 - 85.7)	84 (81 - 86)
Platelets ($x10^9$/L)	232 (187 - 267)	151.5 (128 - 208)	154 (116 - 197)
Creatinine (µmol/L)	85 (76.2 - 102.3)	80 (71 - 100)	79 (67 - 120)
pH		7.37 (7.35 - 7.41)	7.37 (7.34 - 7.38)
BE		-3.5 (-2.2 - -4.8)	-2.8 (-1.8 - -3.7)
Lactate		2.1 (1.6 - 3.5)	1.4 (1 - 2.3)
PaO2/FiO2 (kPa)		31.4 (26.3 - 44.2)	43 (31 - 52)

Table 3-4. Physiological variables and laboratory blood results. Values expressed as Median (IQR). HR, heart rate; MAP, mean arterial pressure; SOFA, Sequential Organ Failure Assessment; WCC, white cell count; BE, Base Excess; kPa, kilopascals. There was no significant difference between the different sample timings (p value >0.05, Kruskal-Wallis ANOVA with Dunn's comparison test).

At 3h post-CPB, two patients needed an intra-aortic balloon pump (IABP), and one of them needed milrinone. At 18 – 24h post-CPB, 8 patients were on

mechanical ventilation and sedated, 4 patients had an IABP and 1 patient was on continuous veno-venous hemofiltration (CVVH). One patient died on day 31.

3.3.3 NOP and ppN/OFQ mRNA expression by PMN leucocytes

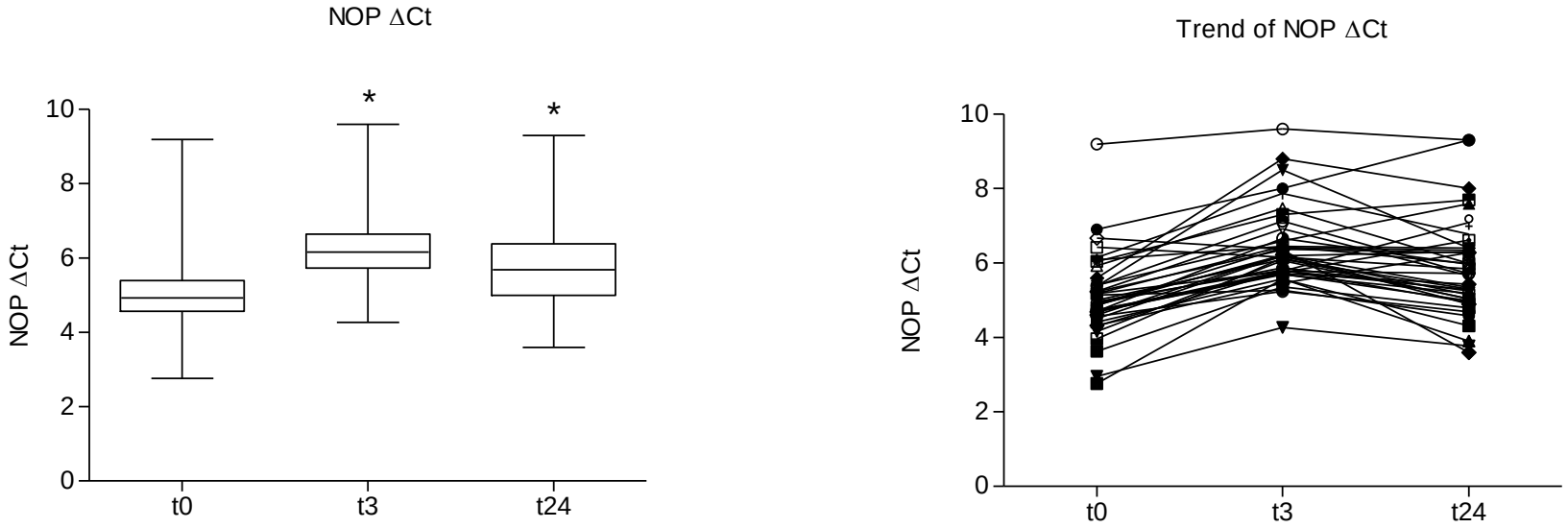
NOP and ppN/OFQ gene expression by polymorphonuclear leucocytes was assessed by changes in ΔCt (difference in cycle threshold between gene of interest and housekeeping gene, GAPDH). An increase of ΔCt represents a reduction in gene expression. We present data for the ΔCt, fold change ($2^{(-\Delta\Delta Ct)}$) and percentage change.

NOP

NOP mRNA expression by polymorphonuclear leucocytes significantly reduced at 3h post-CPB (t3) and remained significantly reduced at 18 – 24h post-CPB (t24). This is expressed by an increase in ΔCt from a median value of 4.9 at t0 to 6.1 at t3 (this one cycle difference is a halving of start material). All but 2 and 6 patients had NOP mRNA reduction at t3 and t24 respectively (**Figure 3-1** and **Figure 3-2**).

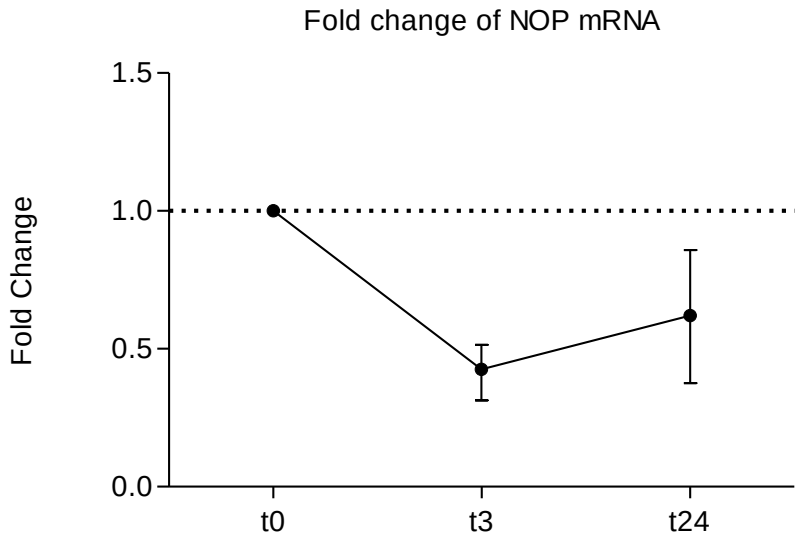
ppN/OFQ

ppN/OFQ mRNA expression by polymorphonuclear leucocytes decreased significantly at t3 and remained significantly lower than baseline at t24. This is expressed by an increase in ΔCt from 13.7 at t0 to 18.2 at t3. **Figure 3-3.** and **Figure 3-4**.



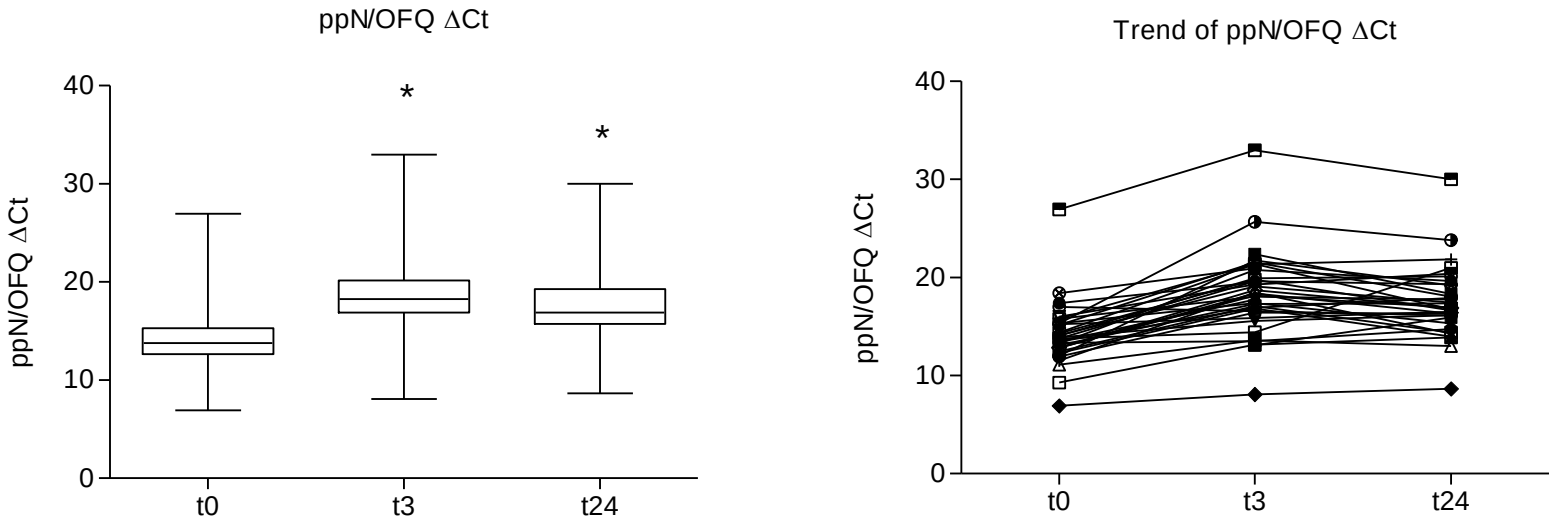
NOP	t0 (n=40)	t3 (n=40)	t24 (n=40)
ΔCt	4.9 (4.5 – 5.3)	6.1 (5.7 - 6.6)*	5.6 (4.9 - 6.3)*

Figure 3-1. NOP mRNA expression by polymorphonuclear leucocytes in patients undergoing cardiac surgery. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Friedman ANOVA with Dunn's multiple comparison test. *p<0.05 compared to induction samples.



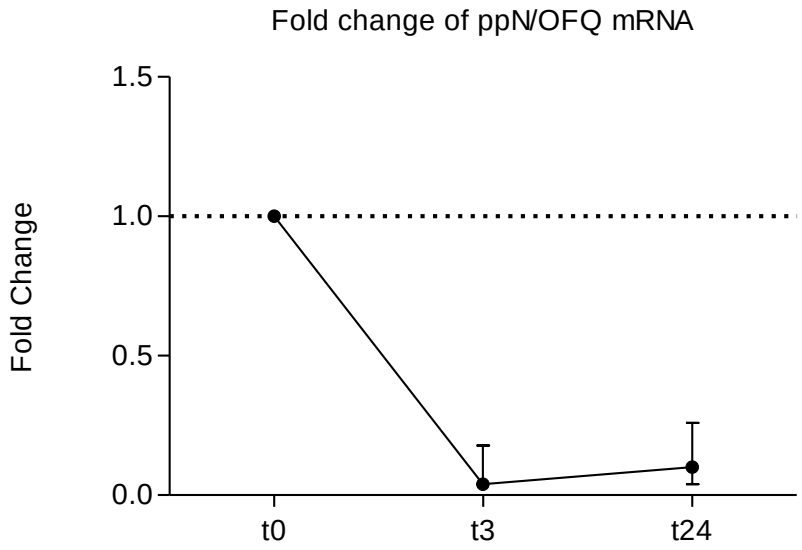
NOP	t3	t24
Fold change	0.42 (0.3 – 0.49)	0.55 (0.33 – 0.8)
% change	↓57% (50% – 68%)	↓44% (19% – 66%)

Figure 3-2. Fold change in NOP mRNA expression by polymorphonuclear leucocytes in patients undergoing cardiac surgery. n=40 at all three sampling times. Data expressed as median (IQR). t3 and t24 are compared to t0.



	t0 (n= 34)	t3 (n= 36)	t24 (n= 34)
ppN/OFQ ΔCt	13.7 (12.7 - 15.3)	18.2 (16.8 - 20.5)*	16.8 (15.7 - 19.2)*
Undetermined ΔCt	n=6	n=4	n=6

Figure 3-3. mRNA expression of ppN/OFQ by polymorphonuclear leucocytes in patients undergoing cardiac surgery. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. *p<0.05 compared to induction samples. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test.



ppN/OFQ	t3 n= 32	t24 n= 31
Fold change	0.04 (0.02 – 0.17)	0.1 (0.04 – 0.26)
% change	↓95% (81% – 98%)	↓91% (74% – 96%)

Figure 3-4. Fold change in ppN/OFQ mRNA expression by polymorphonuclear leucocytes in patients undergoing cardiac surgery. Data expressed as median (IQR) comparing t3 and t24 with t0. 32 patients had ppN/OFQ ΔCt determined both at t0 and t3; all had reduced mRNA expression at t3. 31 patients had ppN/OFQ ΔCt determined both at t0 and t24, 29 of these patients had reduced mRNA expression.

3.3.4 Relationship between NOP and ppN/OFQ mRNA

There was reduced mRNA expression of both N/OFQ receptor and peptide precursor by polymorphonuclear leucocytes. However, there was no correlation between NOP and ppN/OFQ reduction at 3h post-CPB, **Figure 3-5**.

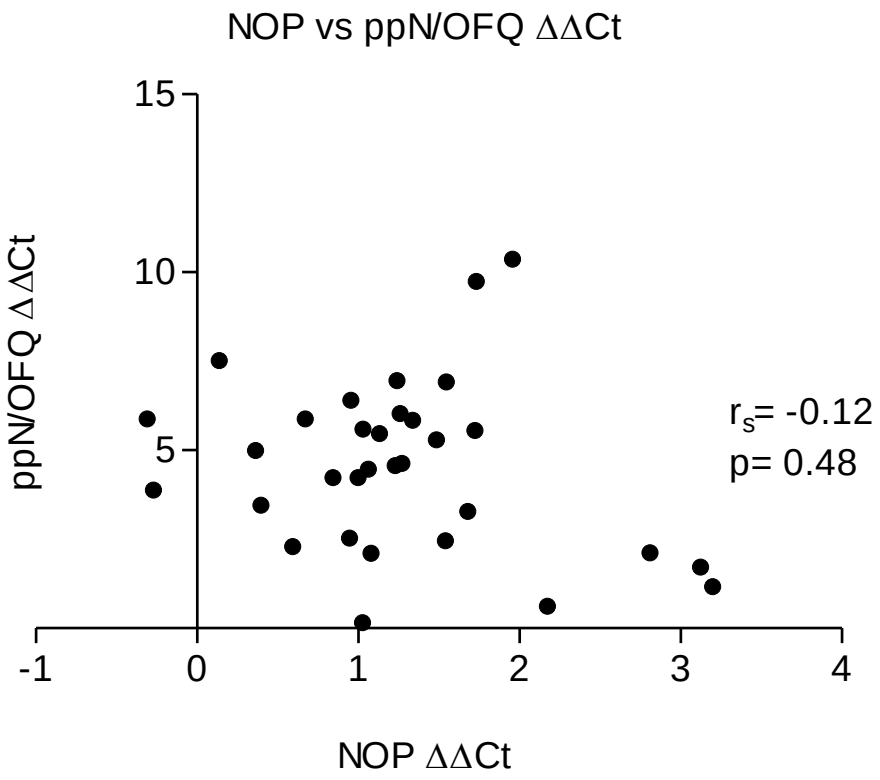
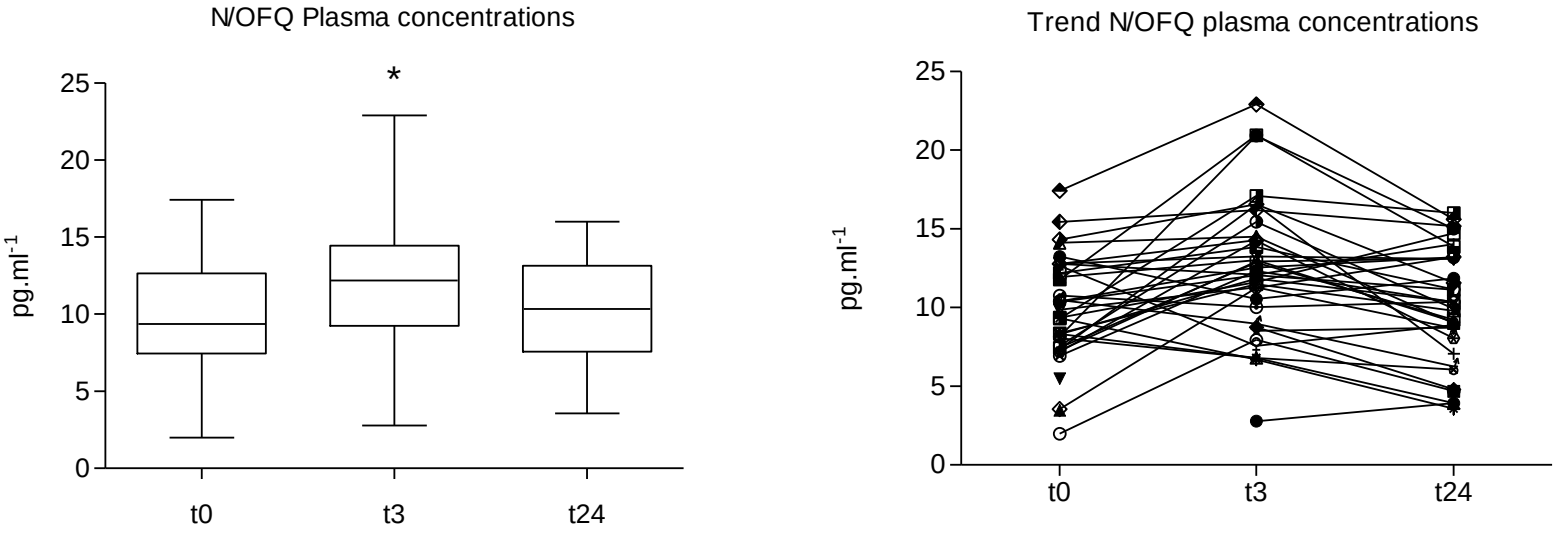


Figure 3-5. Spearman Correlation (r_s) of NOP ΔΔCt vs ppN/OFQ ΔΔCt (ΔCt at t3 minus ΔCt at t0). n=36. There was no correlation between reduced mRNA expression of NOP and reduced mRNA expression of ppN/OFQ at 3h post-CPB.

3.3.5 N/OFQ plasma concentrations measured by RIA

Plasma concentrations of N/OFQ were analysed by radioimmunoassay. Plasma N/OFQ increased significantly from a median value of 9.3 $pg.ml^{-1}$ to 12.2 $pg.ml^{-1}$ at 3h post- CPB, and returned to above baseline concentrations at 18 – 24h post-CPB (median 10.3 $pg.ml^{-1}$), **Figure 3-6**.



Plasma N/OFQ	t0 (n=35)	t3 (n=36)	t24 (n=37)
$pg.ml^{-1}$	9.3 (7.4 – 12.6)	12.2 (9.2 – 14.4)*	10.3 (7.5 – 13.1)

Figure 3-6. Plasma N/OFQ concentrations by RIA during CPB. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Plasma N/OFQ was not determined in 5, 4 and 3 samples at t0, t3 and t24 respectively. In 25/33 patients there was a significant increase at t3 compared to t0, whilst in 8 patients the concentrations were lower. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test. *p=0.01 compared to induction samples.

3.3.6 Relationship between plasma N/OFQ concentrations and NOP and ppN/OFQ mRNA expression

During cardiopulmonary bypass there was an increased plasma concentration of N/OFQ and a reduced expression of NOP and ppN/OFQ mRNA by polymorphonuclear leucocytes. There was an inversely proportional relationship between plasma N/OFQ and NOP mRNA expression. The higher the plasma N/OFQ (ΔN/OFQ = N/OFQ at t3 minus N/OFQ at t0) the lower the NOP mRNA expression (expressed by a high ΔΔCT value. ΔΔCT= ΔCt at t3 minus ΔCt at t0). Regarding ppN/OFQ, there was no correlation between plasma N/OFQ concentration and ppN/OFQ mRNA expression, **Figure 3-7**

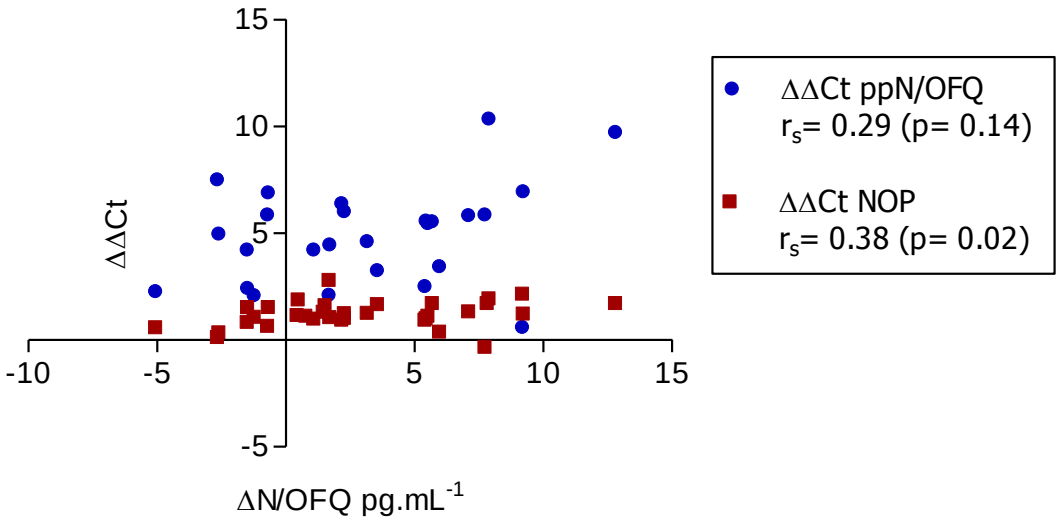


Figure 3-7. Correlation between ΔN/OFQ (t3 minus t0) plasma concentrations and NOP and ppN/OFQ ΔΔCt (ΔCt at t3 minus ΔCt at t0) using Spearman's correlation coefficient.

3.3.7 Inflammatory markers. Cytokines IL-8, IL-10, TNFα, and MPO

IL-8, IL-10, TNFα and MPO concentrations were measured in plasma at t0, t3 and t24.

IL-8

Plasma concentrations of IL-8 were determined in all 3 samples of all 40 patients. In 38 patients IL-8 increased from a median value of 11.6 $pg.ml^{-1}$ at t0 to 91.2 $pg.ml^{-1}$ at t3 (P value <0.01), and still remained significantly higher at t24 (**Figure 3-8**).

IL-10

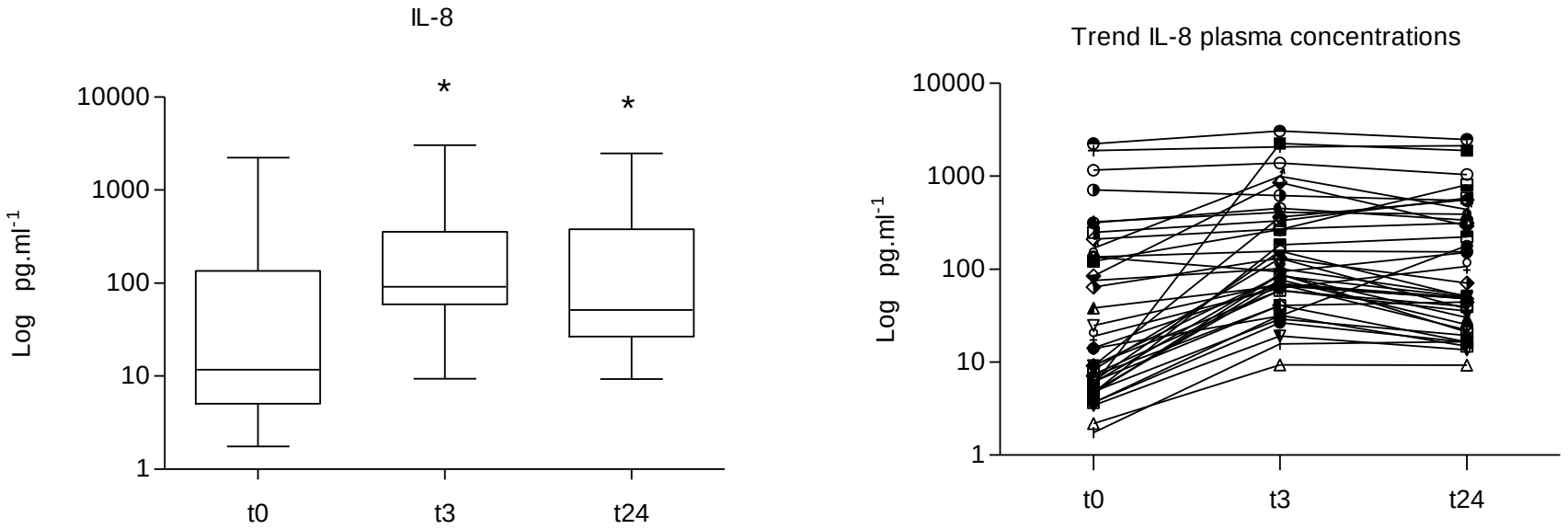
Plasma concentrations of IL-10 were obtained in 32 patients at t0 (other values were below detection limit) and in all 40 patients at t3 and t24. In 30/32 patients there was a significant increase of IL-10 from a median value of 35.4 $pg.ml^{-1}$ at t0 to 164.7 $pg.ml^{-1}$ at t3. At t24 IL-10 returned to above baseline values, 82.2 $pg.ml^{-1}$ (**Figure 3-9)**.

TNFα

Plasma concentrations of TNFα were obtained in 36 patients at t0 and 39 patients at t3 and at t24 (other values were below detection limit). TNFα remained largely unchanged after CPB (**Figure 3-10)**.

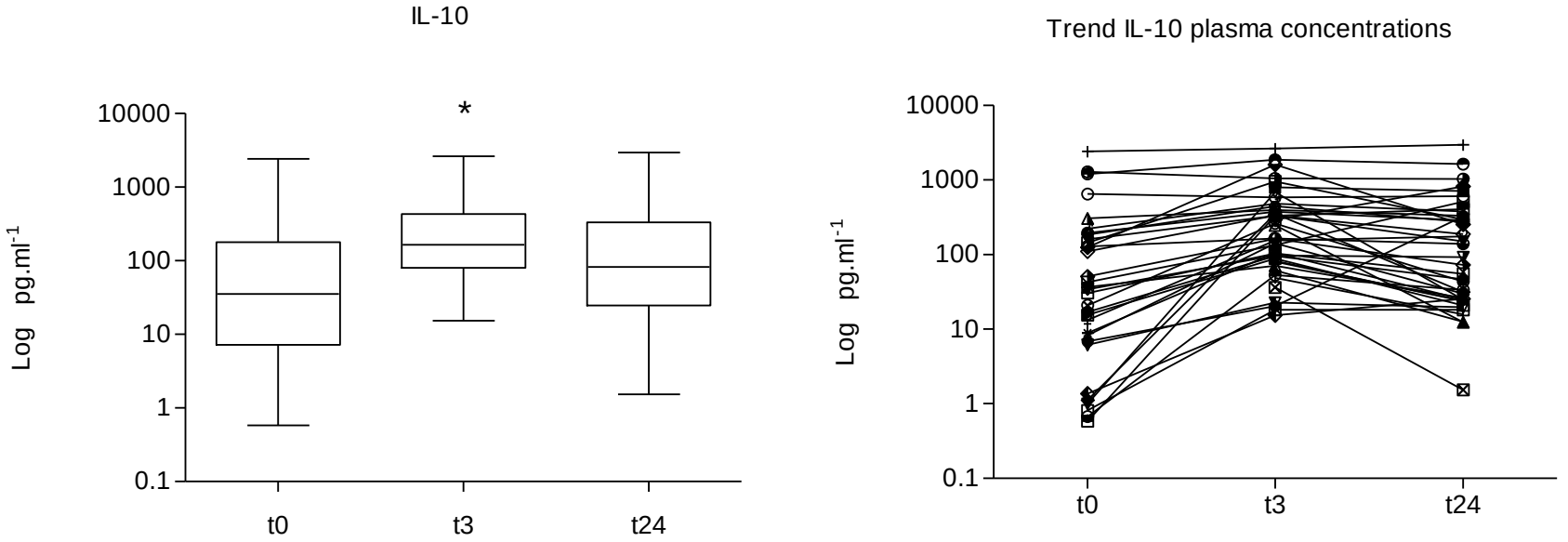
MPO

MPO plasma concentrations were obtained in all 3 samples of all 40 patients. In 37/40 patients MPO significantly increased from 79 $ng.ml^{-1}$ at t0 to 129 $ng.ml^{-1}$ at t3 (p value < 0.0001). At t24 MPO values returned to just below baseline values (**Figure 3-11)**.



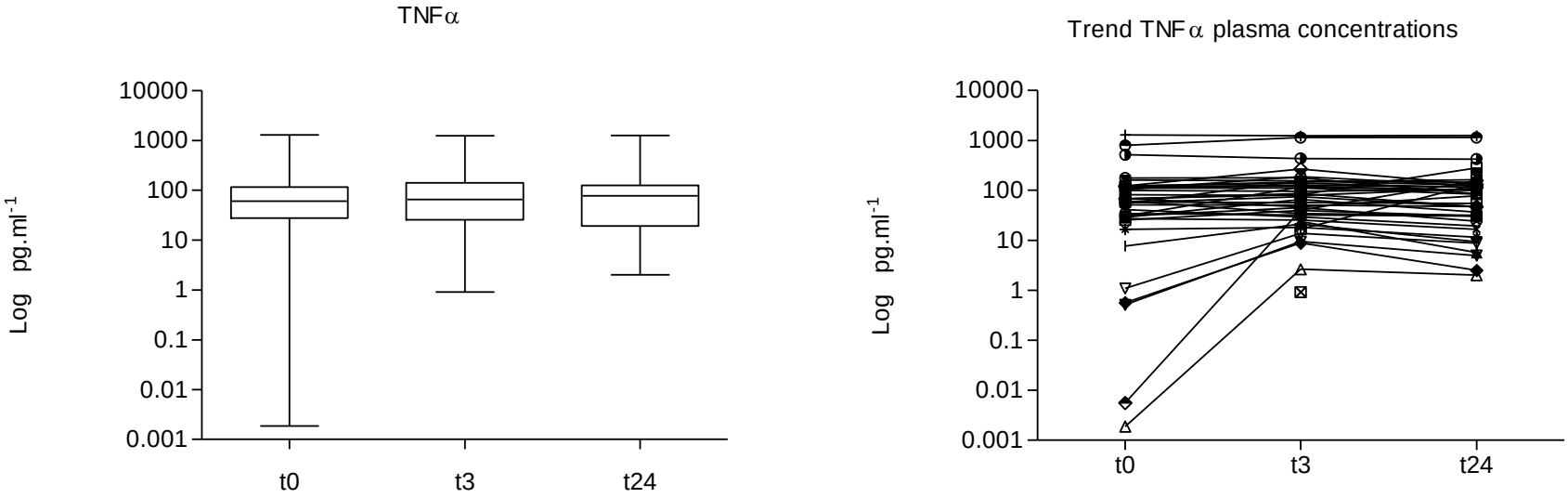
Plasma IL-8	t0 (n=40)	t3 (n=40)	t24 (n=40)
$pg.ml^{-1}$	11.6 (5 - 135)	91.2 (58 - 357)*	51.33 (26 - 377)*

Figure 3-8. IL-8 plasma concentrations in patients undergoing cardiac surgery. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Friedman ANOVA with Dunn's multiple comparison test. *P<0.01



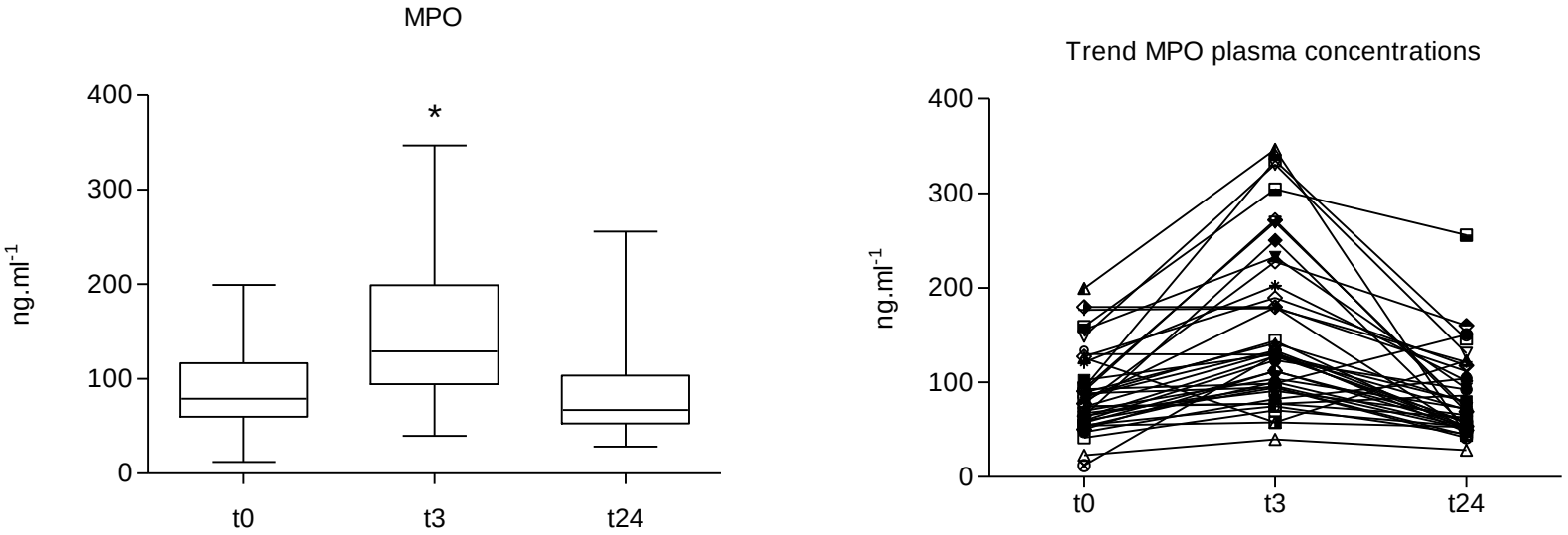
Plasma IL-10	t0 (n=32)	t3 (n=40)	t24 (n=40)
$pg.ml^{-1}$	35.4 (7 - 178)	164.7 (80 - 434)*	82.2 (24 - 332)

Figure 3-9. IL-10 plasma concentrations in patients undergoing cardiac surgery. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test. *P<0.01



Plasma TNFα	t0 (n=36)	t3 (n=39)	t24 (n=39)
$pg.ml^{-1}$	60 (27- 116)	65 (25- 141)	77 (19- 126)

Figure 3-10. TNFα plasma concentrations in patients undergoing cardiac surgery. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test.



Plasma MPO	t0 (n=40)	t3 (n=40)	t24 (n=40)
$ng.ml^{-1}$	79 (59 – 116)	129 (94 – 199)*	67 (52 – 103)

Figure 3-11. MPO Plasma concentrations. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Friedman ANOVA with Dunn's multiple comparison test. *P<0.0001

3.3.8 Relationship between NOP and ppN/OFQ mRNA expression and inflammatory markers during inflammation

There was no relationship between the lower mRNA expression of NOP and ppN/OFQ mRNA during CPB and the inflammatory response measured as increased IL-8, IL-10 and MPO. **Table 3-5** shows spearman correlation and p values.

	ΔΔCt NOP		ΔΔCt ppN/OFQ	
	r_s	p value	r_s	p value
ΔIL-8	0.004	0.9	0.06	0.72
ΔIL-10	0.25	0.16	-0.004	0.98
ΔMPO	0.12	0.43	-0.17	0.35

Table 3-5 Correlation between change in NOP and ppN/OFQ mRNA expression (ΔΔCt (ΔCt at t3 minus ΔCt at t0)) and Δ of inflammatory markers (change of concentrations between t3 and t0) using Spearman's correlation coefficient (r_s).

3.3.9 Relationship between plasma N/OFQ concentrations and inflammatory markers

Plasma N/OFQ concentrations displayed a similar response to CPB as the inflammatory markers IL8, IL10 and MPO, an increase at 3h post-CPB which reduced at 18 – 24h post-CPB. There was a correlation between plasma concentrations of N/OFQ and plasma concentrations of MPO when comparing all 3 samples. However, there was no correlation between concentrations of

plasma N/OFQ and those of IL-8 or IL-10. (**Figure 3-12**, **Figure 3-13**). This may suggest the PMN cell as a common source for N/OFQ and MPO release.

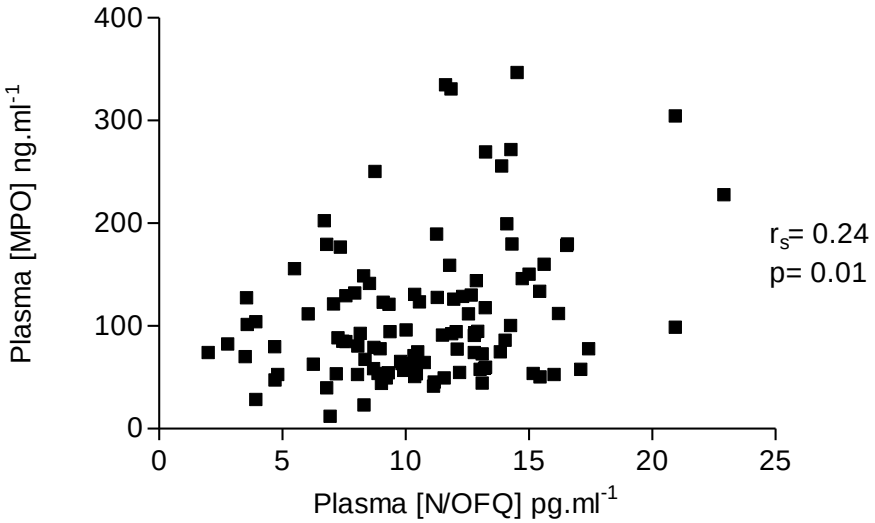
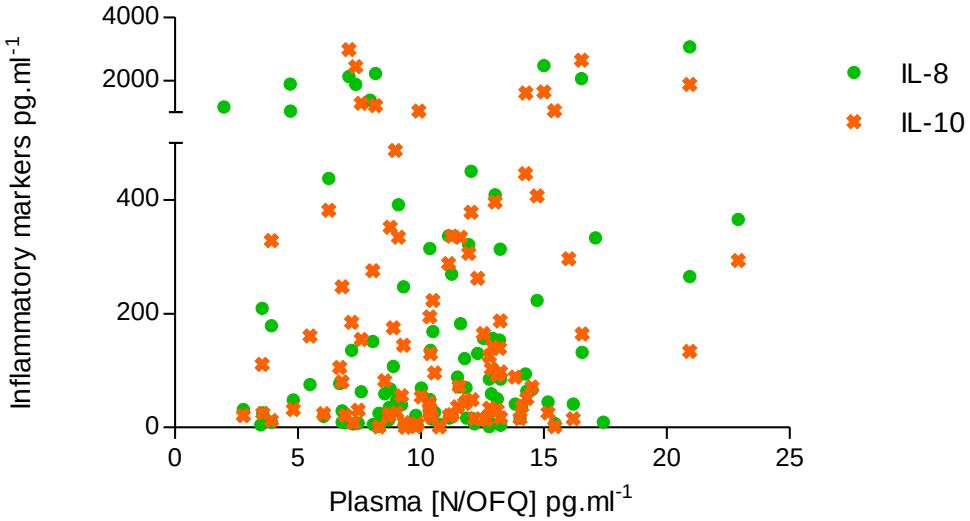


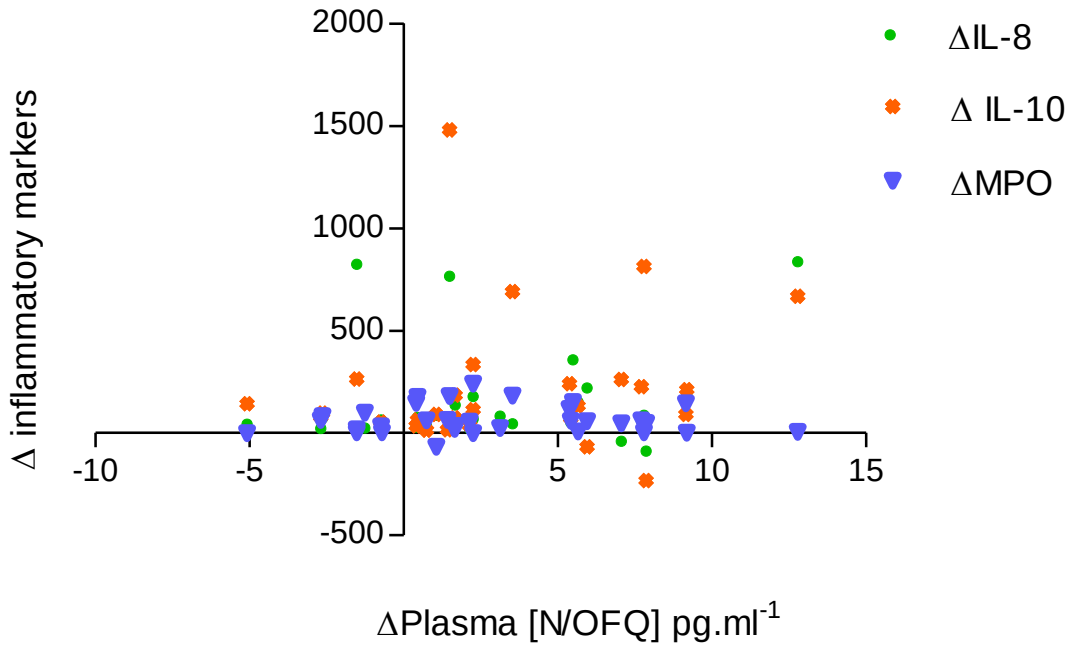
Figure 3-12. Spearman correlation (r_s) of plasma N/OFQ concentrations vs plasma MPO concentrations.



	IL-8	IL-10
r_s	0.13	0.09
P value	0.16	0.33

Figure 3-13. Spearman correlation (r_s) comparing plasma N/OFQ concentrations with plasma IL-8 and IL-10 concentrations.

There was no relationship between the change of IL8, IL10 or MPO (t3 minus t0) with the change seen on plasma N/OFQ concentrations at 3h post-CPB, (t3 minus t0). **Figure 3-14**.



	ΔN/OFQ vs ΔIL-8	ΔN/OFQ vs ΔIL-10	ΔN/OFQ vs ΔMPO
r_s	0.33	0.24	0.003
P value	0.06	0.20	0.98

Figure 3-14. Spearman correlation (r_s) of plasma ΔN/OFQ concentrations (t3 minus t0) vs Δ inflammatory markers (t3 minus t0).

3.3.10 Correlation between clinical findings and laboratory results

We analysed the relationship between plasma N/OFQ concentrations, and mRNA expression of NOP and ppN/OFQ with CPB time, AXC time, and inotropic support. Data showed that plasma N/OFQ concentrations significantly increased at t3 compared to t0, see **Figure 3-15**, and NOP mRNA expression significantly reduced at t3 in patients with longer AXC time, see **Figure 3-16**. CPB time did not have an effect on N/OFQ concentration or mRNA expression of NOP or ppN/OFQ. Neither, there was a correlation between N/OFQ system and inotropic support required.

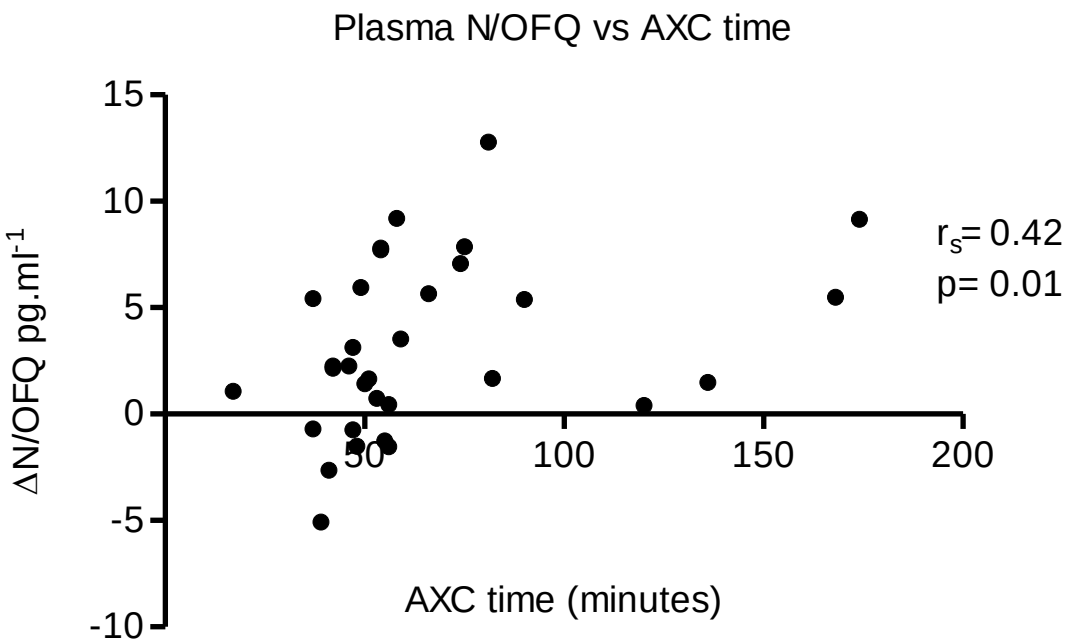


Figure 3-15. Spearman correlation (r_s) of change in plasma N/OFQ concentration (ΔN/OFQ, t3-t0) vs aortic cross clamp (AXC) time.

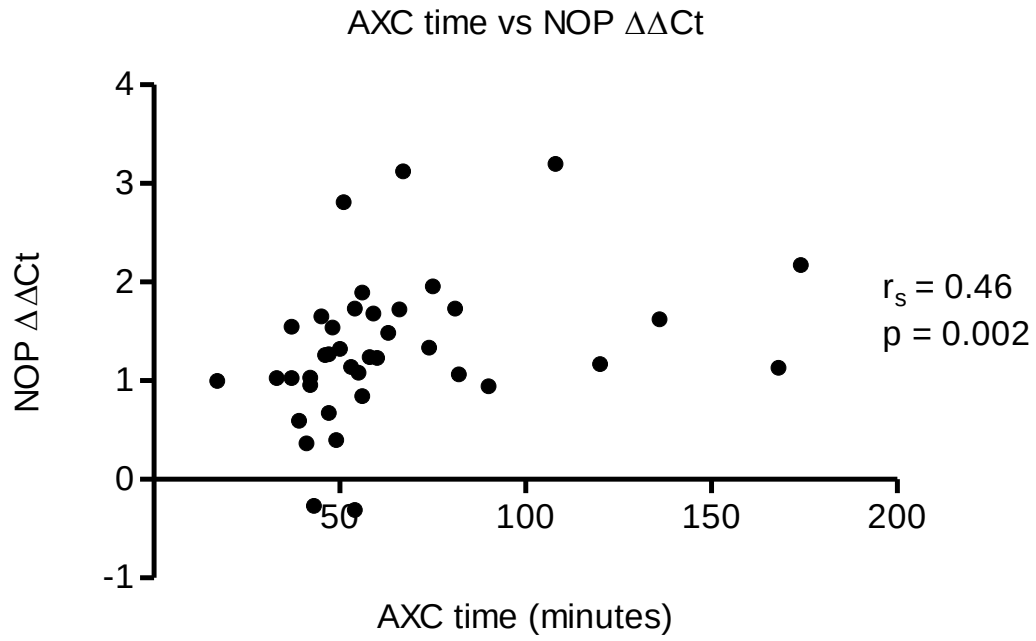


Figure 3-16. Spearman correlation (r_s) of aortic cross clamp time (AXC) vs NOP ΔΔCt (ΔCt at t3 – ΔCt at t0).

3.4 Discussion

These data support the hypothesis that plasma N/OFQ concentrations increase in response to an inflammatory stimulus, and this was inversely related to NOP mRNA expression: the higher the plasma N/OFQ concentration the lower the NOP mRNA expression by PMN cells. We also observed that mRNA expression of NOP and of ppN/OFQ by polymorphonuclear cells is significantly reduced in response to inflammation, contrary to our hypothesis.

In vitro and *in vivo* animal studies have investigated the inflammatory response to central and peripheral administration of N/OFQ. Central administration of N/OFQ in rats undergoing exploratory laparotomy has shown an anti-inflammatory effect via reduced production of inflammatory markers by macrophages.[32] On the contrary, peripheral administration of N/OFQ in anaesthetised[21] and septic[31] rats demonstrated an increase in the inflammatory response by increased leucocyte rolling and adhesion, and macromolecular leak in mesenteric vessels, as well as increased mortality in the septic animals. The animal data available suggests that N/OFQ acts as pro-inflammatory when administered peripherally, leading to increased mortality. These data are further supported by the reduced mortality in response to UFP-101, a NOP antagonist, in septic animals.[31] Despite this, we have not found observational animal data on the behaviour of the N/OFQ system during an inflammatory or septic process, the data available is on response to N/OFQ administration. Studies analysing the N/OFQ expression during SIRS in animals and then the response to a NOP agonist/antagonist are lacking.

The *in vitro* evidence available shows an increased N/OFQ production in rat splenocytes stimulated with pro-inflammatory LPS and Concanavalin A.[29]

In humans, most N/OFQ studies relate to acute and chronic pain, and few studies have been conducted in patients with cardiac disease. Our research group reported that NOP is expressed in the human right atrium of patients with coronary artery disease undergoing cardiac surgery under CPB (n=38), whilst ppN/OFQ mRNA was not expressed (n=10).[11] Whether the expression of NOP is related to the underlying ischaemic heart disease or to the CPB is unknown. Fontana and colleagues[7] measured plasma N/OFQ concentrations in patients with stable and unstable angina. Even though the plasma N/OFQ concentrations were increased in those patients with unstable angina, it is difficult to conclude whether this is related to pain or to ischaemic heart disease itself.

3.4.1 Plasma N/OFQ

We found in this study that plasma N/OFQ concentrations increased in response to the inflammatory stimulus of CPB. N/OFQ behaved in a similar manner to known inflammatory markers such as IL-8, IL-10 and MPO. Williams and colleagues[33] also reported an increase in plasma N/OFQ concentrations in response to sepsis. In Williams' study the higher concentrations of N/OFQ were found in patients who died of sepsis. We have conducted our study in mostly elective patients undergoing cardiac surgery, where physiological and biochemical patient response to CPB was similar amongst patients. Plasma

N/OFQ was found to be increased with prolonged aortic cross clamp time, but not with CPB time.

3.4.2 NOP and ppN/OFQ mRNA expression by PMN cells

PMN cells comprise the majority of the immunocyte cell population and is comprised mainly of neutrophils (42 – 70%).[120] The most common response to inflammation is leucocytosis with neutrophilia, where the neutrophil count can reach over 90% of leucocytes, therefore we opted for analysing the PMN cell population.

mRNA expression of NOP and ppN/OFQ is known to occur in various immune cells, which is not the case for classical opioid receptors (mu: MOP, kappa: KOP, delta: DOP).[16] Monocytes, lymphocytes,[14;15] and PMN cells, [14;17] they all have been reported to express mRNA for the N/OFQ receptor and peptide.

In this study we observed reduced mRNA expression of both the N/OFQ receptor NOP and its peptide precursor ppN/OFQ by PMN cells, contrary to our hypothesis. These findings are partly similar to those of Stamer and colleagues,[34] where there was also a reduced expression of ppN/OFQ mRNA but an increase in NOP mRNA in septic patients. It is clear that they are two different patient populations (SIRS and Sepsis) with different cells being analysed (PMN cells and peripheral blood cells), however one would hope for a similar response by immunocytes to SIRS or Sepsis.

It is possible that when plasma N/OFQ concentrations increase in response to the inflammatory process, ppN/OFQ and NOP mRNA expression fall as they are degraded to produce protein.

To date, there are no other human studies available in the literature observing the response of the N/OFQ system by immunocytes during inflammation.

3.4.3 Limitations of the study

There are some limitations to this study:

1. All PCR studies are limited to investigating the mRNA expression of a given gene; however, this does not necessarily translate into the amount of protein, which is the critical response to analyse. In our study we have measured N/OFQ by RIA. In the absence of any usable specific antibodies and the limited amount of tissue needed for radioligand binding assays we were unable to directly quantify NOP protein.

2. Sampling time in CPB studies vary from either after the start or the end of CPB. We chose to take the first sample at 3 hours after the start of CPB, as this is the closest to the inflammatory stimulus and the inflammatory response is more likely to be at its highest. In addition, there is the practical point that immediate laboratory processing of the samples is needed and this is a lengthy process which is facilitated by earlier sampling.

3. There are no data available relating to N/OFQ and CPB or N/OFQ expression by PMN cells during inflammation. This was performed as a pilot study. The number of 40 patients was chosen as being feasible to recruit with the resources available, including time. The literature shows a great variety of sample sizes in CPB studies, varying from around 15 to 150 patients. We considered 40 patients for a pilot study would give an adequate representation

of the population regarding mRNA expression of the N/OFQ system by PMN cells.

There were no exclusion criteria in this observational study. Adult patients listed for elective or semi-elective cardiac surgery who gave informed consent were recruited, thus reducing patient bias.

3.5 Conclusion

These data confirmed our hypothesis that plasma N/OFQ concentrations in humans would increase in response to an inflammatory stimulus in a similar manner to IL-8, IL-10 and MPO. The source of this peptide during inflammation is unknown: immunocytes being a likely potential source. In this study we found a reduced expression of mRNA precursor ppN/OFQ as well of its receptor NOP; NOP expression was related to higher plasma N/OFQ concentrations. It is possible that immunocytes release N/OFQ during an inflammatory process and this leads to a negative feedback, reducing the expression of NOP and ppN/OFQ from the immune cells. Further studies are needed to investigate the protein expression of N/OFQ by immunocytes; in particular each immune cell type.

4 Effect of N/OFQ *in vitro* on NOP and ppN/OFQ mRNA expression by PMN cells

4.1 Introduction

Preliminary analysis of the mRNA expression of NOP and ppN/OFQ by PMN cells during cardio pulmonary bypass (Chapter 3: The effect of Cardiac surgery under cardiopulmonary bypass on the N/OFQ system), showed reduced mRNA expression of both peptide and receptor at 3 hours after cardiopulmonary bypass.

Previously, our research group found increased plasma N/OFQ concentrations in those critically ill patients with sepsis who died,[33] and we found increased plasma N/OFQ concentrations in response to an inflammatory process such as cardiopulmonary bypass. We, therefore, hypothesised that plasma N/OFQ has a negative feedback effect on mRNA expression of NOP and ppN/OFQ by PMN cells. With this in mind, we exposed PMN cells to N/OFQ *in vitro* and analysed the change in NOP and ppN/OFQ mRNA expression.

4.1.1 Sample collection

Local research ethics committee approval was obtained as detailed in Chapter 3: The effect of Cardiac surgery under cardiopulmonary bypass on the N/OFQ system.

Blood samples (7.5 ml in EDTA sampling bottles) were obtained before induction of anaesthesia from 4 patients (study identification numbers CN18,

20, 23 and 26) undergoing cardiac surgery under cardiopulmonary bypass, and transferred at room temperature to the laboratory.

4.1.2 Method

PMN cell extraction

PMN cell extraction was performed by gradient method using Polymorphprep™ (Axis-Shield), as described in Chapter 2: Methods, following the manufacturer's protocol.

Incubation

PMN cells were suspended in 1 ml of PBS. The cells were divided into two groups:

- N/OFQ group: PMN cells + 1 µM N/OFQ (concentration chosen as top of the dose response curve for most *in vitro* pharmacological measures) + 10 µM peptidase inhibitor to prevent peptide breakdown during the experiment. These were amastatin, bestatin, phosphorhamidon and captopril.
- Control group: PMN cells + 10 µM peptidase inhibitor

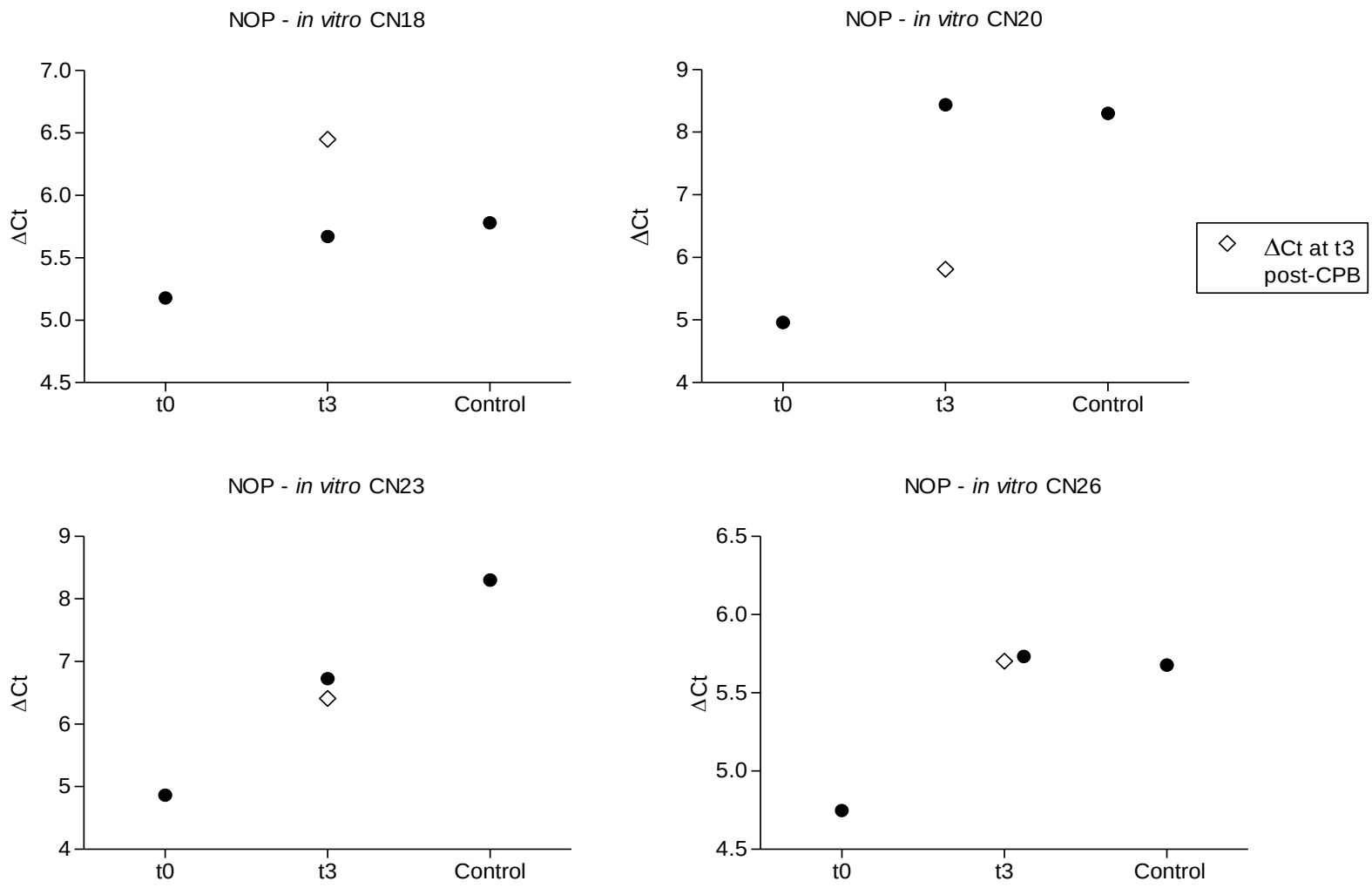
The samples were incubated at 37˚C for 3 h, which was the time the second sample was taken in the CPB study described in Chapter 3, 3 hours after start of CPB. .

After 3 h of incubation the cells were centrifuged and the pellet dissolved in 1 ml of Tri-reagent and stored at -80˚C for RT-PCR batch analysis (described in Chapter 2: Methods).

4.1.3 Results

NOP

After 3 hours incubation there was reduced NOP mRNA expression in all 4 patients' samples. However, the control group with only peptidase inhibitor had similar reduced mRNA expression as the N/OFQ *in vitro* group. See **Figure 4-1**.

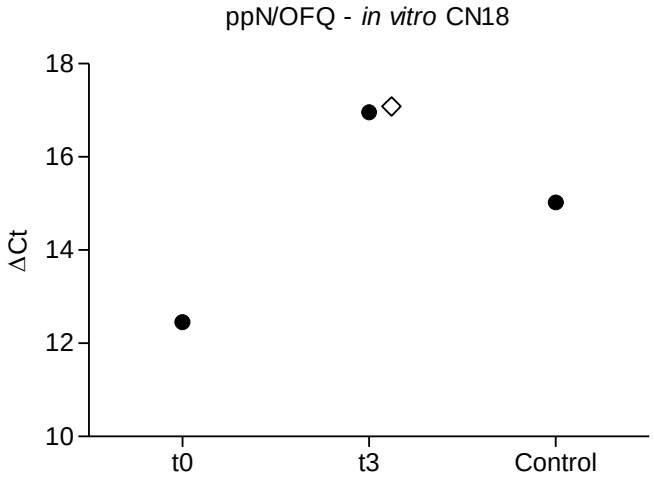

NOP - *in vitro* CN18
ΔCt
7.0
6.5
6.0
5.5
5.0
4.5
t0
t3
Control
NOP - *in vitro* CN20
ΔCt
9
8
7
6
5
4
t0
t3
Control
ΔCt at t3 post-CPB
NOP - *in vitro* CN23
ΔCt
9
8
7
6
5
4
t0
t3
Control
NOP - *in vitro* CN26
ΔCt
6.5
6.0
5.5
5.0
4.5
t0
t3
Control

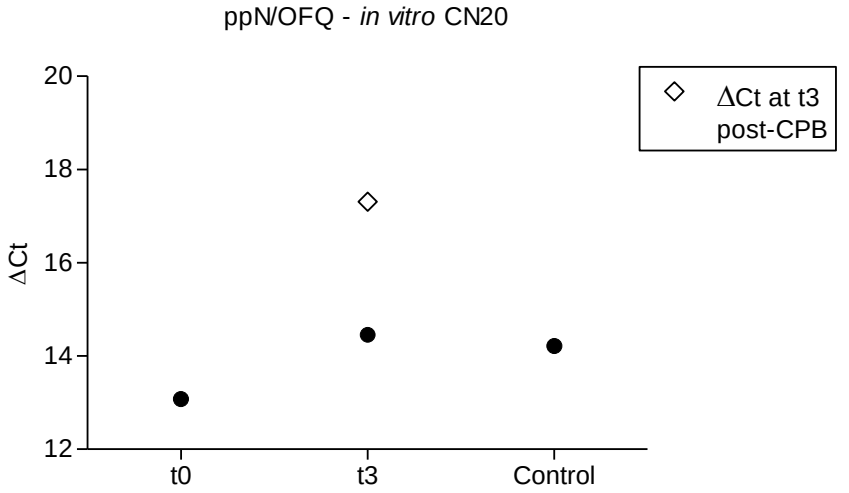
NOP	t0 ΔCt	t3 *in vitro* ΔCt (% change)	Control *in vitro* ΔCt (% change)	t3 ΔCt Post-CPB (% change)
CN 18	5.1	5.6 (↓28%)	5.7 (↓34%)	6.4 (↓58%)
CN 20	4.9	8.4 (↓91%)	8.3 (↓90%)	5.8 (↓44%)
CN 23	4.8	6.7 (↓72%)	6.6 (↓71%)	6.4 (↓65%)
CN 26	4.7	5.7 (↓49%)	5.6 (↓47%)	5.7 (↓48%)

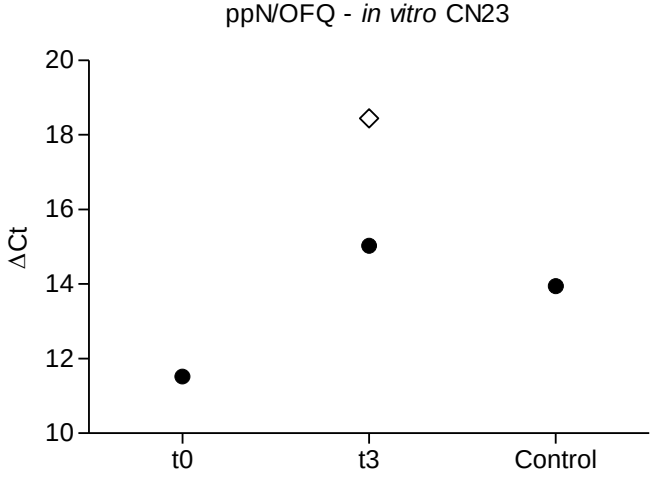
Figure 4-1 NOP ΔCt of in vitro PMN cells from 4 patients undergoing cardiopulmonary bypass. t0: induction of anaesthesia; t3 *in vitro*: 3 h post-incubation on N/OFQ; Control *in vitro*: 3 h post-incubation on peptidase inhibitor. In brackets % change of mRNA expression of NOP compared to t0. An increase in ΔCt represents a reduction in mRNA expression (↓). ◊ indicates ΔCt at 3 h post-CPB, for comparison.

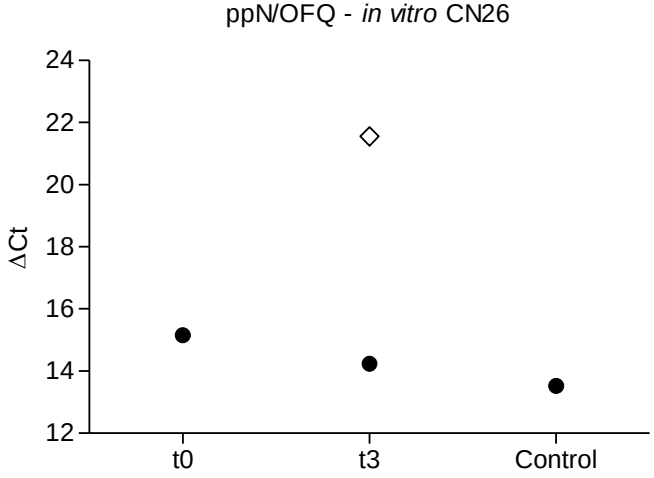
ppN/OFQ

After 3 hours incubation of PMN cells with N/OFQ, ppN/OFQ mRNA expression was reduced in 3 out of 4 patients' samples. Similar effect was seen in the control peptidase inhibitor only group. Comparing ppN/OFQ expression at 3 hours post-CPB and post-*in vitro* N/OFQ, there was higher reduction in mRNA expression during CPB in 3 patients. See **Figure 4-2**.


ppN/OFQ - *in vitro* CN18
ΔCt
18
16
14
12
10
t0
t3
Control


ppN/OFQ - *in vitro* CN20
ΔCt
20
18
16
14
12
t0
t3
Control
ΔCt at t3 post-CPB


ppN/OFQ - *in vitro* CN23
ΔCt
20
18
16
14
12
10
t0
t3
Control


ppN/OFQ - *in vitro* CN26
ΔCt
24
22
20
18
16
14
12
t0
t3
Control

ppN/OFQ	t0 ΔCt	t3 *in vitro* ΔCt (% change)	Control *in vitro* ΔCt (% change)	t3 ΔCt Post-CPB (% change)
CN 18	12.4	16.9 (↓95%)	15 (↓83%)	17 (↓95%)
CN 20	13	14.4 (↓61%)	14.2 (↓54%)	17.3 (↓94%)
CN 23	11.5	15 (↓91%)	13.9 (↓81%)	18.4 (↓99%)
CN 26	15.1	14.2 (↑88%)	13.5 (↑209%)	21.5 (↓98%)

Figure 4-2 ppN/OFQ ΔCt of in vitro PMN cells from 4 patients undergoing cardiopulmonary bypass. t0: induction of anaesthesia; t3 *in vitro*: 3 h post-incubation on N/OFQ; Control *in vitro*: 3 h post-incubation on peptidase inhibitor. In brackets % change of mRNA expression of ppN/OFQ compared to t0. An increase in ΔCt represents a reduction in mRNA expression (↓) and vice versa. ◊ indicates ΔCt at 3 h post-CPB, for comparison.

4.2 Discussion

Exposure of PMN cells to N/OFQ *in vitro* showed reduced expression of NOP and ppN/OFQ mRNA, similar to the effect seen *in vivo* after 3 hours of CPB. However, the control *in vitro* samples, with only peptidase inhibitor, had similar mRNA expression response to N/OFQ as the N/OFQ samples.

From this small sample size we cannot draw any firm conclusions as to whether N/OFQ is responsible for the reduced mRNA expression of NOP and ppN/OFQ seen after CPB. The effect seen by peptidase inhibitor (PI) may be due to PI protecting any released N/OFQ from the PMN cell and therefore potentiating any autocrine feedback effect.

Further studies are needed in both healthy volunteers and patients, to quantify the mRNA expression of PMN cells exposed to different doses of N/OFQ. In addition other methods of peptide protection should be considered such as using a range of more selective peptidase inhibitors.

4.3 Conclusion

The data obtained from this *in vitro* study suggests the possibility that increased plasma N/OFQ concentration has an effect on mRNA expression of the N/OFQ system by PMN cells. In this small *in vitro* study we found reduced NOP and ppN/OFQ mRNA expression by PMN cells when exposed to N/OFQ 1 µM. However, a similar response was obtained within the control group containing Peptidase Inhibitor only. These results either suggest that *in vitro* N/OFQ has no effect on mRNA expression of NOP / ppN/OFQ or that the peptidase inhibitor potentiates the effect of any N/OFQ released by the PMN cell itself.

5 The effect of systemic sepsis on the N/OFQ system

5.1 Introduction

In the period between 1995 and 2000, 27% of the Intensive Care Unit (ICU) admissions in the UK were due to severe sepsis.[121] Of these patients 35% died in the ICU and 47% during their hospital stay. There has been an increase in the number of patients admitted to ICU with sepsis, from 23% in 1996 to 28% in 2004.[122] Even though hospital mortality decreased from 48% in 1996 to 44% in 2004, the overall number of deaths increased.

The definitions of SIRS (systemic inflammatory response syndrome), sepsis, severe sepsis, and septic shock, that describe the clinical response and provide a working definition for clinical studies, were initially defined in the consensus conference by the American College of Chest Physicians and the Society of Critical Care Medicine in 1991,[123] and have subsequently been adapted or modified to make them more clinically applicable.[124] **Table 5-1**

Condition	Definition
SIRS	Systemic inflammatory response to a variety of severe clinical insults. The response is manifested by two or more of the following conditions: 1) Temperature >38°C or <36°C 2) Heart rate >90 bpm 3) Respiratory rate >20 breaths per minute or PaCO2 <4.2 kPa 4) White blood cell count >12 x 10^9/l or <4 x 10^9/l, or >10% immature forms
Sepsis	SIRS in response to infection, manifested by two or more of the above conditions + evidence of infection.
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion may be manifested by lactic acidosis, oliguria, acute alteration in mental status, acute lung injury, cardiac dysfunction, amongst others.
Septic shock	Severe sepsis associated with hypotension despite adequate fluid resuscitation or need of inotropic support to maintain mean BP > 60mmHg.

Table 5-1 Definitions of SIRS and sepsis.[123;124]

The pathophysiology of sepsis involves multiple inter-related pathways. One of the initial processes of cellular immunity involves activation of leucocytes, which triggers a sequence of events: neutrophil rolling and adhesion to activated endothelium; migration of leucocytes into the affected tissues by chemotaxis; recognition and binding to micro-organisms; and finally engulfment and phagocytosis of microorganisms. These processes are mediated by

cytokines (tumour necrosis factor and interleukins IL-1, IL-2, IL-6, IL-8, IL-18) released from macrophages, monocytes, and other cells, which maintain the ongoing chemotaxis and inflammatory response.[53]

This inflammatory response is controlled by programmed cell death or apoptosis of leucocytes, which seems to be delayed in sepsis. If the leucocyte-mediated response is not limited to the zone of initial infection, a widespread inflammatory response occurs. Moreover, neutrophils also contain proteolytic enzymes and produce reactive oxygen species, which are necessary for the degradation of engulfed pathogens. If these substances, along with inflammatory cytokines are released, then damage to the surrounding healthy tissue can occur.[125] Mitochondrial-specific oxidative damage is a potential treatment target to prevent the inflammatory response and multi-organ failure.[126]

Consequences of sepsis include: 1) dysfunction of the macro and microcirculation which compromises tissue perfusion and organ function; 2) hypotension and vascular dysfunction secondary to increased nitric oxide (NO) production, activation of vascular potassium channels and hormonal changes (e.g. vasopressin and cortisol); and 3) metabolic dysfunction such as hyperglycaemia which leads to widespread cellular damage.[53]

Various inflammatory markers and scoring systems have been investigated and used in the critically ill patient with sepsis to assist with diagnosis, severity of

the disease, and prognosis. Increased CRP, PCT, IL-6, IL-8, IL-10 and TNFα plasma concentrations relate to severity of sepsis.[127-129]

APACHE II and SOFA score are used to predict the outcome of ICU patients. SOFA score on the first day of bacteraemia was shown to be an independent prognostic factor for outcome.[130]

5.1.1 N/OFQ system and sepsis

Various *in vitro* and *in vivo* studies have shown the presence of NOP and N/OFQ in immune cells and its involvement during an inflammatory and septic process. At present there are only two human studies available in the literature in patients with sepsis, observing the response of plasma N/OFQ[33] and that of NOP and ppN/OFQ mRNA expression by peripheral blood cells.[34] This has been explained in detail in Chapter 1: Introduction.

5.2 Materials and methods

5.2.1 Patients recruitment and sample collection

With research ethics committee approval (REC ref: 09/H0722/21) fifty one critically ill patients with a clinical diagnosis of sepsis admitted to the Intensive Care Unit at the Leicester Royal Infirmary were recruited between August 2009 and May 2011. Written consent or relative's assent was obtained either prospectively or retrospectively as appropriate. Two patients were withdrawn from the study due to inability to obtain consent or assent, leaving 49 patients in total.

Inclusion criteria were: patients who were admitted with sepsis, (SIRS + infection, according to the definition given by the ACCP/SCCM), in the previous 24 hours or who developed sepsis in the ICU during the 24 hours before recruitment. Clinical and laboratory data were collected at each sampling time. Blood samples were taken mainly from the indwelling arterial catheter; in few cases where the arterial line was not present blood samples were taken from the central venous catheter.

We aimed to obtain 3 blood samples where feasible:

1. Day 1 sample (D1): sample obtained within 24 hours of admission or of developing sepsis

2. Day 2 sample (D2): sample obtained 18 – 24 hours after the first blood sample.

3. Recovery sample (Rec): sample obtained once the patient had clinically recovered from the septic episode, did not have SIRS and all routine laboratory

markers of inflammation or infection had returned to normal. This sample was obtained in 22/49 patients at 7 to 134 days post-recruitment (median [IQR]= 16 [9.7 – 41.2] days). The recovery sample was used as a baseline value and was taken in either the hospital or at home. These 22 patients comprise the recovery sub-group.

5.2.2 RT-PCR

Polymorphonuclear leucocytes were isolated from 15 ml of blood sample and RT-PCR for NOP and ppN/OFQ mRNA expression performed. Details of polymorphonuclear extraction, mRNA extraction using *miRvana*™ isolation kit and RT-PCR are described in Chapter 2: Methods.

5.2.3 Blood collection for plasma extraction

As described in Chapter 2: Methods, 7.5 ml of blood was aspirated from the arterial line into an EDTA bottle. 150 µl of aprotinin (containing 4.5 TIU, 0.6 $TIU.ml^{-1}$) were immediately added to prevent protein degradation. The samples were then transferred on ice to the laboratory and centrifuged at 3,000x g, 4˚C for 15 minutes. The plasma obtained is stored in 1ml aliquots at -80˚C pending use in batch analysis of N/OFQ peptide by radioimmunoassay, PCT and cytokines and MPO by ELISA.

5.3 Statistical analysis

GraphPad Prism® 5 was used for statistical analysis. Data were assumed to have non-parametric distribution. PCR results on D1, D2 and Recovery were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test

where appropriate. Spearman's Correlation coefficient for non-parametric continuous data was used to determine if there was a correlation between laboratory data (PCR results and inflammatory markers) and physiological data (e.g. inotropic support, white cell count, lactate concentrations, and survival from sepsis).

5.3.1 Study power

This was a pilot observational study and therefore no prospective power calculation. During study design, data collection and analysis, there were no published data on gene expression in a similar patient population on which to base a power calculation. Recently however, Stamer and colleagues[34] published a PCR study on the expression of NOP and ppN/OFQ by peripheral blood cells in 18 critically ill patients with sepsis and found higher NOP expression compared to healthy controls. Notwithstanding the limitations of retrospective power calculations, a retrospective power calculation based on our results showed that: in order to detect a difference in PCR of 1 cycle in ppN/OFQ and in NOP mRNA expression with a power of 80% and an alpha error 0.05, a sample size of 52 and 12 patients respectively would be required.

5.4 Results

Baseline patient characteristics are shown in **Table 5-2**. Patients had mixed co-morbidities and the source of sepsis was mainly either chest or abdomen. Other sources of sepsis involved neutropenic sepsis, necrotising fascitis, and central venous catheter sepsis.

5.4.1 Baseline patient characteristics

Age (years)	65 (49 – 72)
Male/Female (n)	24/25
Weight (Kg)	74 (63 – 92)
Source of sepsis (n (%))	
Chest	18 (36)
Abdomen	23 (47)
Other	8 (16)
APACHE II score	20 (15 – 23)
Co-morbidities (n (%))	
Hypertension	17 (34)
IHD	4 (8)
Hypercholesterolemia	5 (10)
Diabetes	7 (14)
COPD/Asthma	7 (14)
Hypothyrioidism	3 (6)
Chronic kidney disease	2 (4)
Cancer	11 (22)
ITU stay (days)	6 (3 – 10)
30 day outcome (n (%))	
Hospital	17 (34)
Home	19 (39)
Dead	13 (26)
1 year mortality (n (%))	17 (35)
Predicted mortality (%(IQR%))	40 (25 - 40)

Table 5-2 Patient characteristics. n=49. Values expressed as median (Interquartile range, IQR) or number (%). 1 year mortality includes 13 patients who died within 30 days. One patient died at 9 months, and 3 patients at 2 months post-recruitment. Predicted mortality based on APACHE score.

5.4.2 Physiological variables

Physiological variable	D1 n=49	D2 n=46
Temperature (˚C)	37.8 (37 – 38.5)	37.2 (36.7 – 37.5)
MAP (mmHg)	78 (68 – 89)	79 (72 – 85)
HR	110 (97 – 118)	95 (87 – 108)
Creatinine ($\mu mol.l^{-1}$)	125 (81 – 176)	116 (60 – 179)
WCC ($x10^{9}.l^{-1}$)	13.1 (6.3 – 19.7)	13.5 (7.8 – 13.4)
Neutrophils (%)	87 (79 – 91)	88 (82 – 91)
CRP $mg.l^{-1}$	205 (128 – 277) n= 33	223 (164 – 284) n= 36
SOFA score	6 (4 – 8)	5 (3 – 7)
PaO_2/FiO_2 (kPa)	30 (17 – 45)	41 (23 – 53)
Platelet count ($x10^{9}.l^{-1}$)	213 (105 – 358)	203 (135 – 288)
Hb ($g.dl^{-1}$)	9.7 (8.5 – 10.5)	9.4 (8.7 – 10.9)
INR	1.4 (1.3 – 1.7)	1.3 (1.2 – 1.6)
ALT ($IU.l^{-1}$)	27 (19 – 53)	26 (18 – 38)
Albumin ($g.l^{-1}$)	20 (14 – 24)	21 (16 – 24)
Arterial blood gases:		
pH	7.32 (7.26 – 7.37)	7.34 (7.30 – 7.40)
Base deficit	6.2 (3.2 - 8.6)	5.1 (1.9 – 7.5)
Lactate	2.2 (1.2 – 3.3)	1.6 (1.1 – 2.4)
<u>Inotropes</u>		
Noradrenaline ($\mu g.kg^{-1}.min^{-1}$)	0.24 (0.09 – 0.43) n=29	0.19 (0.11 – 0.27) n=21
Adrenaline	n=2	n=2
<u>Ventilation</u>		
Spontaneous	7 (14)	8 (17)
NIPPV	11 (22)	7 (15)
IPPV	31 (63)	31 (67)

Table 5-3 Physiological variables on D1 and D2. 3 patients died before D2 sample. Values expressed as median (Interquartile range, IQR) or number (%).

5.4.3 Recovery sub-group patient characteristics, n=22

Age (years)	70 (45-72)
Male/Female (n)	10/12
Weight (Kg)	76 (66 – 97)
Source of sepsis (n (%))	
Chest	8 (36)
Abdomen	11 (50)
Other*	3 (13)
APACHE II score	19 (15 – 20)
Co-morbidities (n (%))	
Hypertension	9 (41)
IHD	2 (9)
Hypercholesterolemia	2 (9)
Diabetes	3 (14)
COPD/Asthma	3 (14)
Hypothyroidism	1 (4)
Chronic kidney disease	1 (4)
Cancer	4 (4)
ITU stay (days)	6.5 (5 – 9.5)
30 day outcome (n (%))	
Hospital	8 (36)
Home	13 (59)
Dead	1 (4)

Table 5-4. Characteristics of patients with a recovery sample. n=22. Values expressed as median (Interquartile range, IQR) or number (%).
*Sepsis from cellulitis (n=2) and from central line (n=1).

5.4.4 Physiological variables, recovery sub-group n=22

Physiological variable	D1	D2	Recovery
Temperature (˚C)	38.2 (37.5 – 38.8)	37.2 (36.7 – 38)	37 (36.5 – 37.2)
MAP (mmHg)	75 (66 – 92)	78 (74 – 84)	82 (76 – 92)
HR	110 (99 – 123)	90 (83 – 106)	95 (75 – 101)
Creatinine ($\mu mol.l^{-1}$)	130 (80 – 183)	128 (70 – 193)	59 (44 – 81)
WCC ($x10^9.l^{-1}$)	15.4 (10.3 – 19.9)	16.9 (9.5 – 23.0)	9 (7.5 – 10.9)
Neutrophils (%)	88 (82 – 93)	89 (83 – 92)	73 (60 – 81)
CRP $mg.l^{-1}$	233 (159 – 289)	268 (213 – 307)	37 (17 – 84)
SOFA score	6 (5 – 8)	4 (3 – 7)	
PaO_2/FiO_2 (kPa)	32 (23 – 46)	44 (27 – 53)	
Platelet count ($x10^9.l^{-1}$)	244 (162 – 425)	227 (147 – 403)	397 (282 – 582)
Hb ($g.dl^{-1}$)	9.8 (8.5 – 10.9)	9.4 (8.7 – 10.9)	9.8 (8.3 – 11.9)
INR	1.4 (1.3 – 1.7)	1.3 (1.2 – 1.6)	1.1 (1.1 – 1.2)
ALT ($IU.l^{-1}$)	26 (21 – 45)	25 (18 – 38)	33 (20 – 43)
Albumin ($g.l^{-1}$)	23 (15 – 28)	21 (17 – 24)	33 (29 – 36)
ABG			
pH	7.33 (7.28 – 7.37)	7.34 (7.30 – 7.37)	
Base deficit	6.2 (0.7 – 8.4)	5.1 (1.8 – 7.5)	
Lactate	1.5 (1.2 – 3.0)	1.4 (1 – 2.5)	
<u>Inotropes</u>			
Noradrenaline ($\mu g.kg^{-1}.min^{-1}$)	0.2 (0.08 – 0.39) n=13	0.13 (0.08 – 0.23) n= 9	

Table 5-5 Physiological variables on D1, D2 and Recovery from sepsis for 22 patients. Values expressed as median (Interquartile range, IQR). ABG, arterial blood gases. ABG were not obtained with the recovery sample as not clinically indicated.

5.4.5 Patient characteristics and physiological variables of 13 patients who died of sepsis

Thirteen out of 49 patients died within 17 days of being admitted to ICU with sepsis. Patient characteristics and physiological findings of these patients are shown on **Table 5-6** and **Table 5-7**.

Age (years)	63 (51 – 68)
Male/Female (n)	7/6
Weight (Kg)	74 (63 – 92)
Source of sepsis (n)	
Chest/ Abdomen/ Other*	4/ 6/ 3
APACHE II score	21 (18 – 29)
Co-morbidities (n)	
Hypertension	3
IHD	1
Hypercholesterolemia	1
Diabetes	3
COPD/Asthma	2
Hypothyrioidism	1
Chronic kidney disease	1
Cancer	4
Mortality days[§]	6 (2 – 9)

Table 5-6 Characteristics of patients who died of sepsis. n=13. Values expressed as median (Interquartile range, IQR) or number. *Neutropenic sepsis [§]Number of days from ICU admission to death.

Physiological variable	D1 n=13	D2 n=10
Temperature (˚C)	37.4 (36 – 37.9)	37.1 (36.7 – 37.3)
MAP (mmHg)	75 (64 – 85)	82 (68 – 85)
HR	103 (96 – 112)	98 (88 – 117)
Creatinine ($\mu mol.l^{-1}$)	132 (93 – 265)	139 (92 – 234)
WCC ($x10^9.l^{-1}$)	7.2 (1.5 – 14.6)	8.7 (6 – 12)
Neutrophils (%)	89 (73 – 93)	89 (80 – 93)
CRP $mg.l^{-1}$	185 (133 - 236)	201 (147 - 208)
SOFA score	7 (4.5 – 11.5)	6.5 (5.5 – 12)
PaO_2/FiO_2 (kPa)	21 (14 - 34)	24 (15 - 36)
Platelet count ($x10^9.l^{-1}$)	163 (52 – 310)	149 (49 – 208)
Hb ($g.dl^{-1}$)	9.7 (8.6 – 10.1)	9.1 (8.1 – 10.3)
INR	1.7 (1.3 – 1.9)	1.3 (1.2 – 1.4)
ALT ($IU.l^{-1}$)	24 (13 – 90)	19 (13 – 257)
Albumin ($g.l^{-1}$)	20 (13 – 25)	22 (16 – 24)
ABG		
pH	7.26 (7.2 – 7.35)	7.33 (7.24 – 7.38)
Base deficit	6.5 (2.7 - 10)	5.2 (3.6 – 8.4)
Lactate	2.7 (1.1 – 3.9)	2.3 (1.6 – 3.6)
Inotropes		
Noradrenaline ($\mu g.kg^{-1}.min^{-1}$)	0.31 (0.12 – 0.59) n=9	0.26 (0.15 – 0.43) n=6

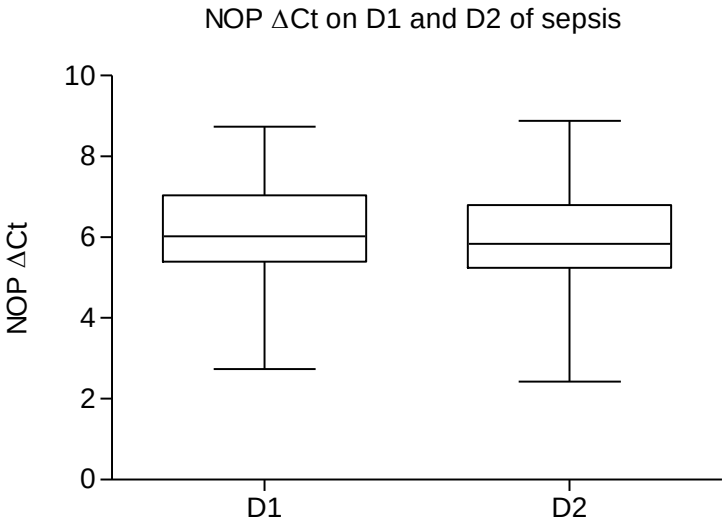
Table 5-7 Physiological variables on D1 and D2 of patients who died of sepsis. 3 patients died before D2 sample could be taken. Values expressed as median (Interquartile range, IQR). ABG, arterial blood gases

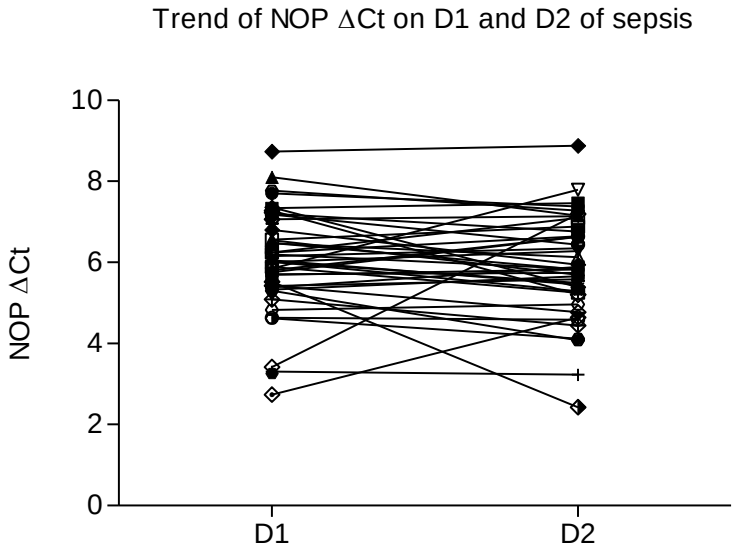
5.4.6 NOP and ppN/OFQ mRNA expression by Polymorphonuclear leucocytes

Expression of the NOP and ppN/OFQ gene by polymorphonuclear cells was assessed by qRT-PCR. The cycle threshold (Ct) and the ΔCt, difference in between Ct for the gene of interest and house keeper GAPDH, were used to determine change in expression. Blood samples were taken on day 1 (D1, n=49), day 2 (D2, n=46) and on recovery from sepsis (Rec, n=22). Three patients died before the second blood sample was due. Even though the recovery sample was taken last, here we present the results for recovery data first to facilitate comparison, and compare ΔCt on D1 and D2 of sepsis with ΔCt in recovery, in 22 patients.

NOP mRNA expression remained similar on D1 and D2 of sepsis, (ΔCt on D1= 6, and on D2=5.8). In the recovery sub-group there was a lower mRNA expression of NOP on day 1 (ΔCt D1=5.9) compared to the recovery control sample (ΔCt Rec=5.2), which was not statistically significant. **Figure 5-1** and **Figure 5-2**

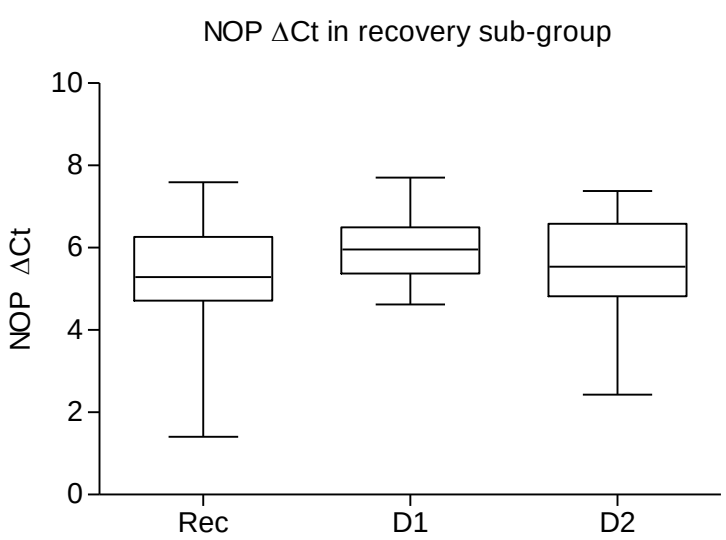
ppN/OFQ mRNA expression was also similar on D1 and D2 of sepsis, (ΔCt on D1=16.5 and on D2=15.7). In the recovery sub-group there was a significant reduction in ppN/OFQ mRNA expression during sepsis compared to the control sample (ΔCt D1=16.8, Rec=13.6). **Figure 5-3** and **Figure 5-4.**

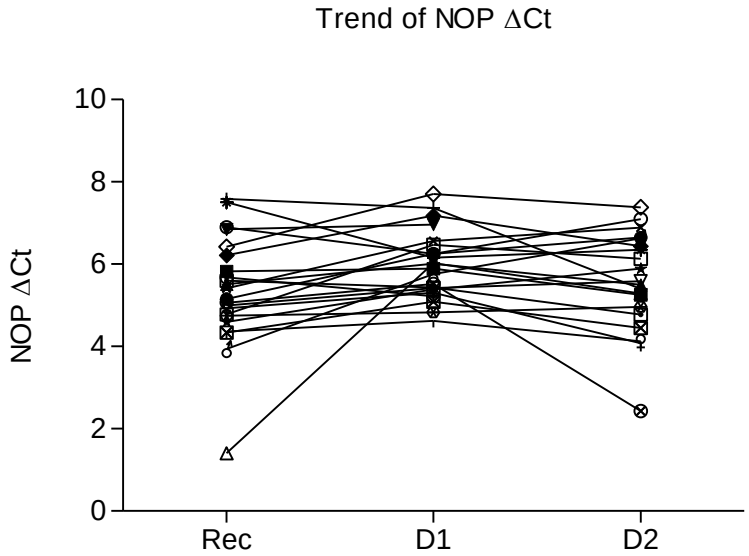




	D1 (n=48)	D2 (n=42)
NOP ΔCt	6.0 (5.3 – 7.0)	5.8 (5.2 – 6.7)

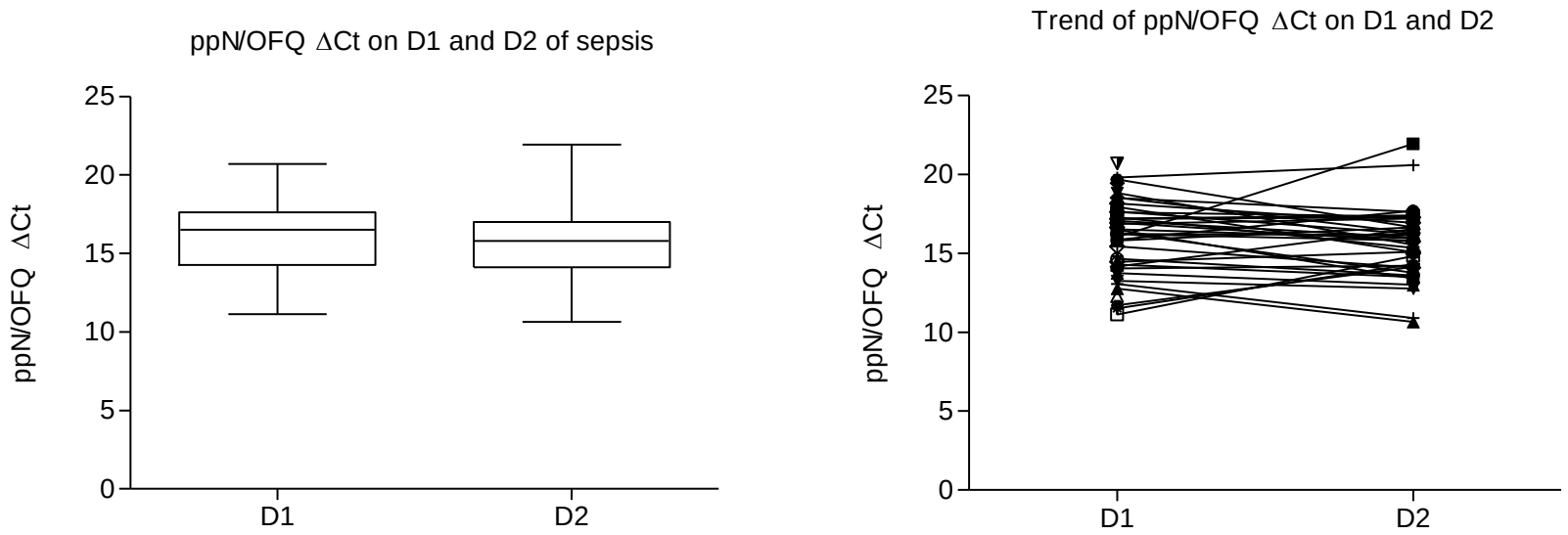
Figure 5-1 NOP mRNA expression by polymorphonuclear leucocytes in patients with sepsis. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. NOP was not determined on 1 and 7 patients on D1 and D2 respectively. There was no statistically significant difference in between days 1and 2 of sepsis (p=0.4). Data were analysed using paired Student's t test.





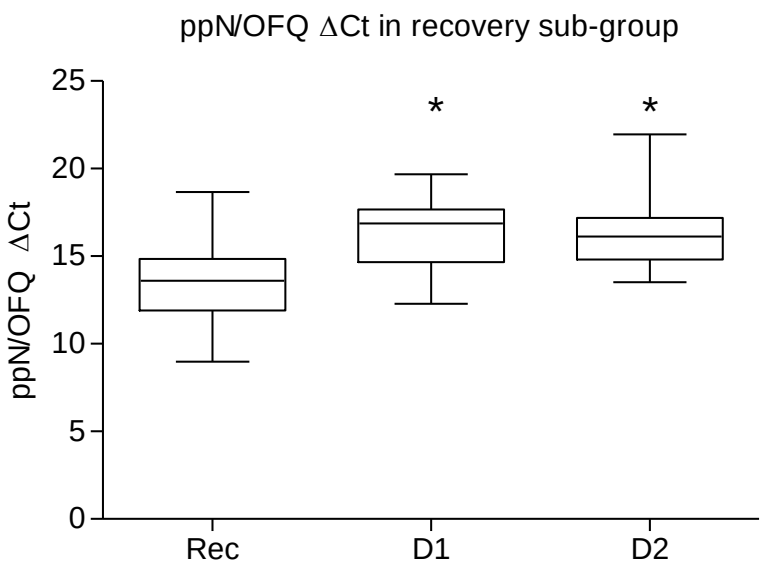
	Rec (n=22)	D1 (n=22)	D2 (n=20)
NOP ΔCt	5.2 (4.7 – 6.2)	5.9 (5.3 – 6.4)	5.5 (4.8 – 6.5)

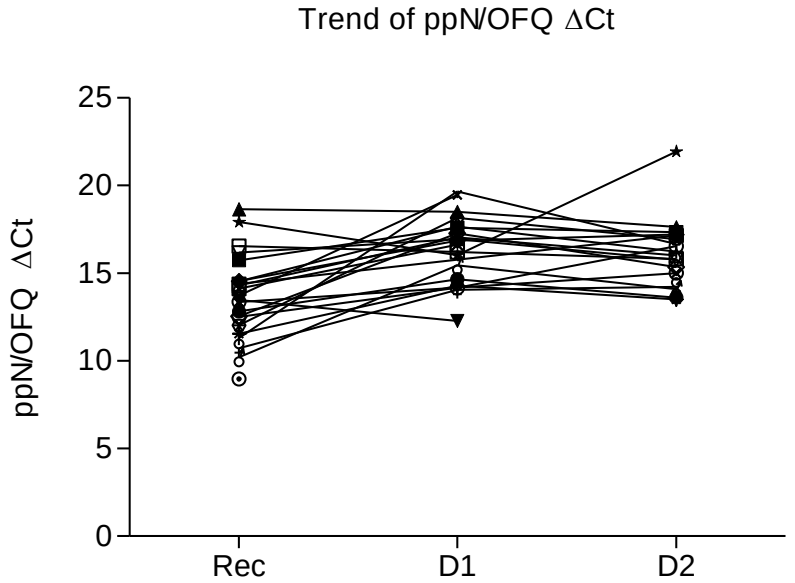
Figure 5-2 NOP mRNA expression in the recovery sub-group in patients with sepsis. In 2 out of 22 patients NOP ΔCt was undetermined on day 2. There was a trend towards lower mRNA expression of NOP (higher ΔCt) on D1 compared to recovery sample with no statistical significance (p= 0.37). Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test. Results for Recovery samples shown first to facilitate comparison with D1 and D2.



	D1 (n=43)	D2 (n=38)
ppN/OFQ ΔCt	16.5 (14.2 – 17.6)	15.7 (14.1 – 17)

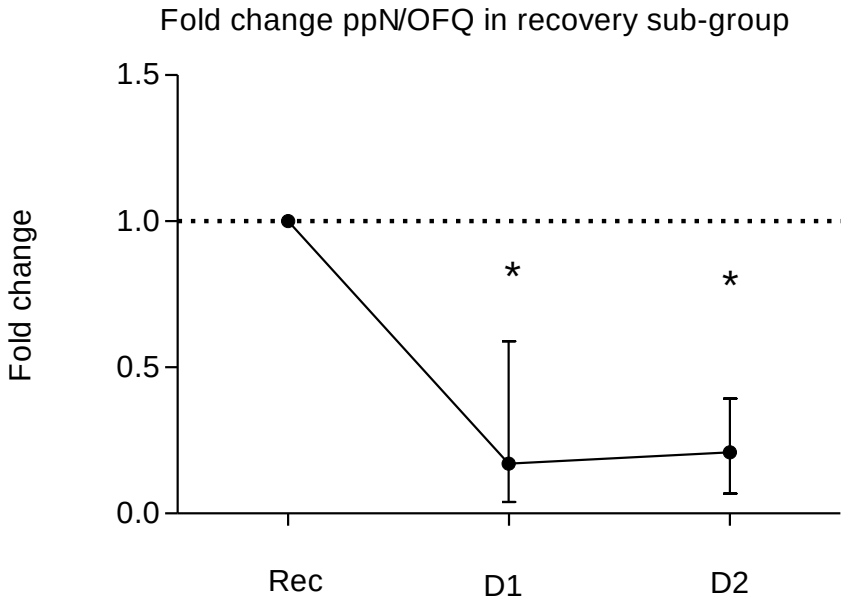
Figure 5-3 ppN/OFQ mRNA expression by polymorphonuclear leucocytes in patients with sepsis. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. ppN/OFQ was determined on 43 and 38 patients on D1 and D2 respectively. There was no significant difference in between the 2 samples (p=0.3). Data were analysed using paired Student's t test.





	Rec (n=22)	D1 (n=19)	D2 (n=18)
ppN/OFQ ΔCt	13.6 (11.9 – 14.8)	16.8 (14.6 – 17.6)*	16.1 (14.8 – 17.1)*

Figure 5-4 ppN/OFQ mRNA expression in 22 patients with sepsis who had D1, D2 and Recovery (Rec) samples taken. There was a significant increase in ppN/OFQ ΔCt (lower mRNA expression) on D1 and D2 compared to recovery sample, *p<0.005. Data expressed as median (boxes and values in parentheses are IQR), end of whiskers indicate range. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test.



ppN/OFQ	D1 (n=19)	D2 (n=18)
Fold change	0.17 (0.04 – 0.59)	0.21 (0.06 – 0.39)
% change	↓85% (72% – 97%)	↓85% (69% – 93%)

Figure 5-5 Fold change of ppN/OFQ mRNA expression by PMN leucocytes in the recovery sub-group of patients with sepsis. D1 and D2 values are compared to Recovery (Rec) values. Data expressed as median (boxes on graph and values in parentheses are IQR), error bars indicate range. All but 4 and 3 patients had ppN/OFQ mRNA reduction at D1 and D2 respectively. There was an 85% reduction in mRNA expression at D1 and D2. *$p<0.005$ compared to Rec sample. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test.

5.4.7 Relationship between NOP and ppN/OFQ mRNA expression

There was significant reduction of ppN/OFQ mRNA expression by polymorphonuclear leucocytes during sepsis, however, there was no correlation between NOP and reduced ppN/OFQ mRNA expression on Day 1 of sepsis compared to Recovery sample, **Figure 5-6**.

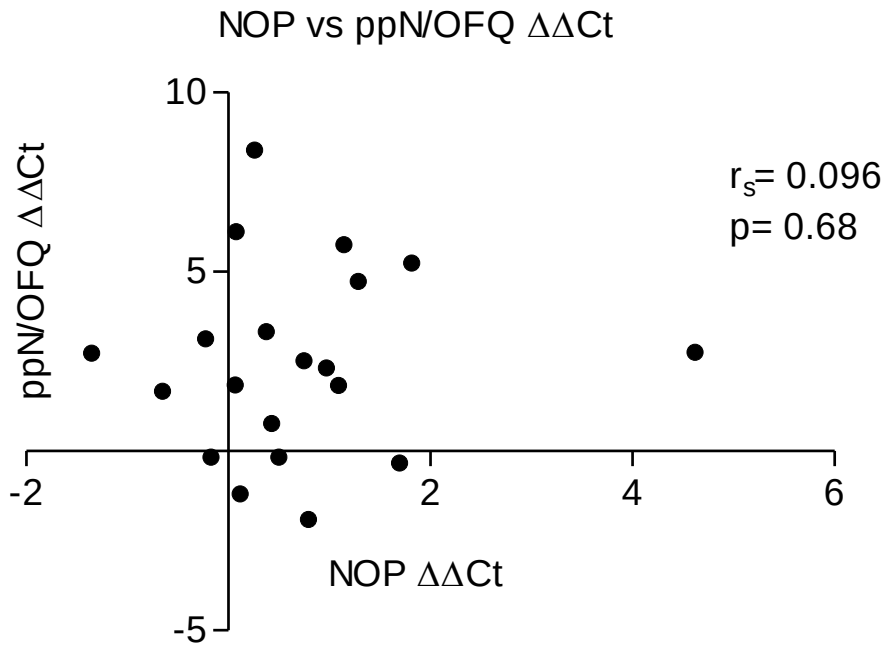


Figure 5-6 Spearman Correlation (r_s) of NOP ΔΔCt vs ppN/OFQ ΔΔCt (ΔCt at D1 minus ΔCt at Rec). n=22. There was no correlation between the change in NOP and ppN/OFQ mRNA expression on day 1 of sepsis compared to recovery.

5.4.8 Relationship between clinical findings and mRNA expression of NOP and ppN/OFQ

We analysed the relationship between NOP and ppN/OFQ mRNA expression with various demographic and physiological parameters on Day 1 of sepsis. **Table 5-8**. Correlation coefficients are simply tests of linear association between two variables, and are limited by the data distribution and the presence of outliers. Performing

multiple correlations increases the chance of a Type 1 error (false positives), whilst adjusting the p values for multiple correlations increases the chance of a Type 2 error (false negatives).[131] We have analysed various correlation coefficients to simply test the possible association with markers of clinical sepsis in a series of observational pilot study. It is controversial whether adjusting the p value is valid and therefore was not performed in this study.

Data show that the higher the white cell count and the percentage of neutrophils, the higher the mRNA expression of NOP (expressed by a lower ΔCt), **Figure 5-7** and **Figure 5-8**. ppN/OFQ mRNA expression on Day 1 of sepsis was lower (higher ΔCt) in those patients who required higher doses of Noradrenaline and in those with a higher plasma lactate concentration. **Figure 5-9** and **Figure 5-10**. To confirm whether lactate, noradrenaline or other variables are associated with NOP or ppN/OFQ, further studies will be needed.

	NOP ΔCt D1(n=48)		ppN/OFQ ΔCt D1 (n=43)	
	r_s (95% CI)	p value	r_s (95% CI)	p value
Age	0.043 (-0.25 to 0.33)	0.76	-0.099 (-0.39 to 0.21)	0.52
Sex	0.212 (-0.08 to 0.47)	0.14	-0.120 (-0.41 to 0.19)	0.44
Source of sepsis	0.052 (-0.24 to 0.33)	0.72	0.204 (-0.11 to 0.48)	0.18
APACHE	-0.110 (-0.39 to 0.18)	0.45	-0.050 (-0.35 to 0.26)	0.75
SOFA score	0.272 (-0.02 to 0.52)	0.06	0.178 (-0.13 to 0.46)	0.25
Noradrenaline use	0.206 (-0.09 to 0.47)	0.16	0.344 (0.03 to 0.59)	**0.02**
Creatinine	-0.07 (-0.35 to 0.22)	0.63	0.013 (-0.29 to 0.32)	0.93
WCC	-0.343 (-0.57 to -0.05)	**0.01**	-0.225 (-0.49 to 0.08)	0.14
%Neutrophils	-0.310 (-0.55 to -0.01)	**0.03**	-0.001 (-0.31 to 0.31)	0.99
Lactate	0.162 (-0.13 to 0.43)	0.27	0.305 (-0.003 to 0.56)	**0.04**
ITU stay days	0.035 (-0.25 to 0.32)	0.81	-0.081 (-0.38 to 0.23)	0.60
Mortality at 30 days	0.114 (-0.18 to 0.39)	0.43	0.000 (-0.30 to 0.30)	1.00

Table 5-8 Correlation between NOP and ppN/OFQ ΔCt on D1 of sepsis with demographic and physiological variables. NOP and ppN/OFQ were undetermined in 1 and 6 patients on D1 respectively. Data were analysed using non-parametric Spearman correlation (r_s). 12 variables were correlated. CI, confidence interval.

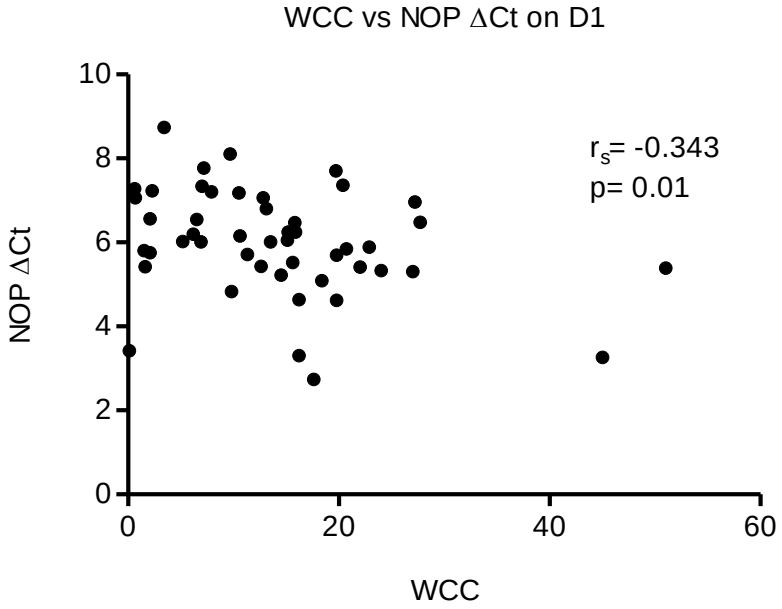


Figure 5-7 Correlation between white cell count (WCC) and NOP ΔCt on D1 of sepsis. n= 48, NOP ΔCt was undetermined in 1 sample. Data were analysed using non-parametric Spearman correlation (r_s)

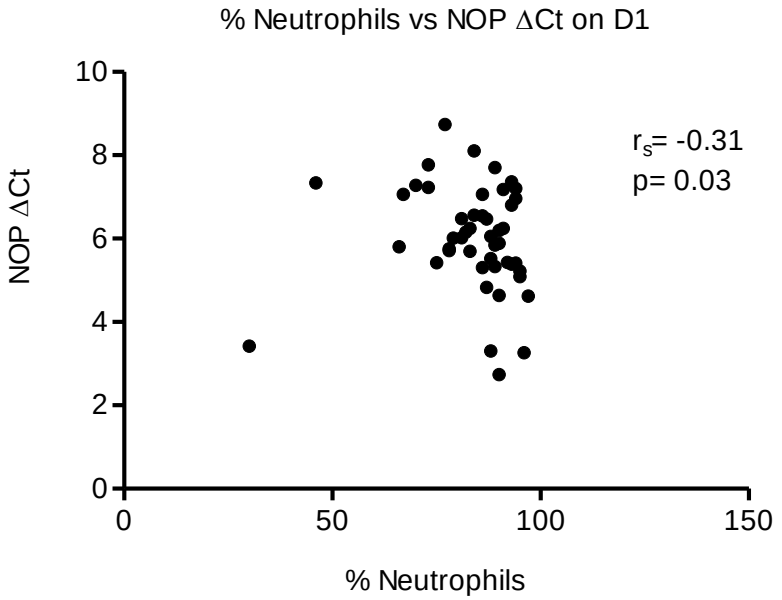


Figure 5-8 Correlation between % of Neutrophils and NOP ΔCt on D1 of sepsis. n= 48, NOP ΔCt was undetermined in 1 sample. Data were analysed using non-parametric Spearman correlation (r_s)

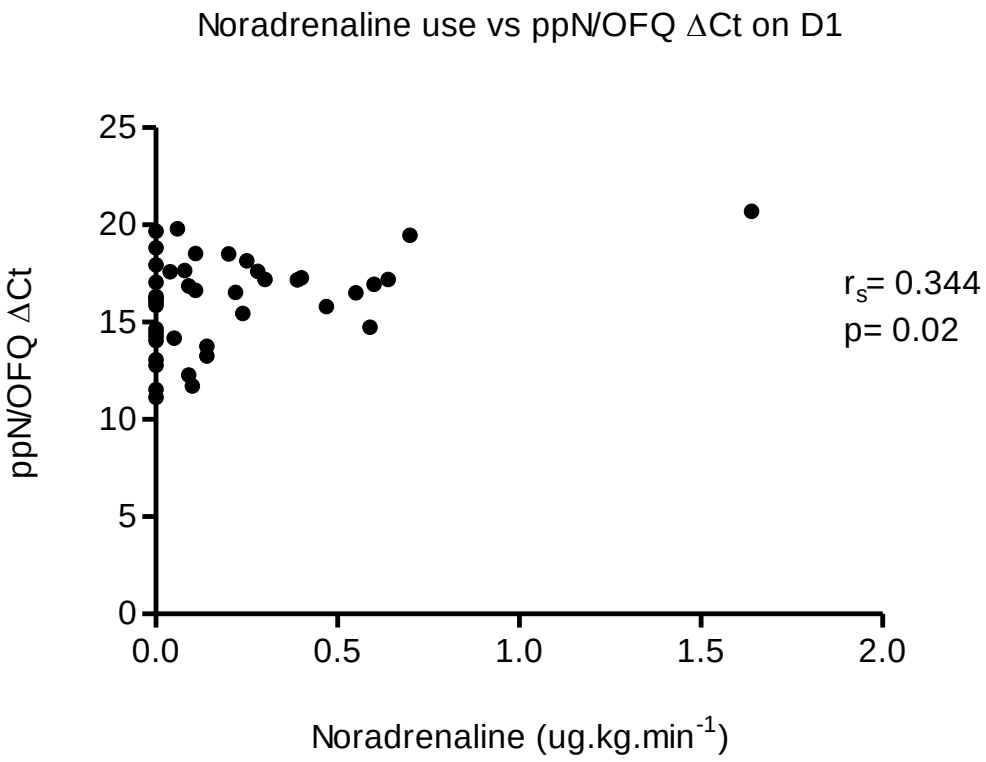


Figure 5-9 Correlation of Noradrenaline requirements vs ppN/OFQ ΔCt on Day 1 of sepsis. n=43, ppN/OFQ mRNA was undetermined in 6 samples. Data were analysed using non-parametric Spearman correlation (r_s)

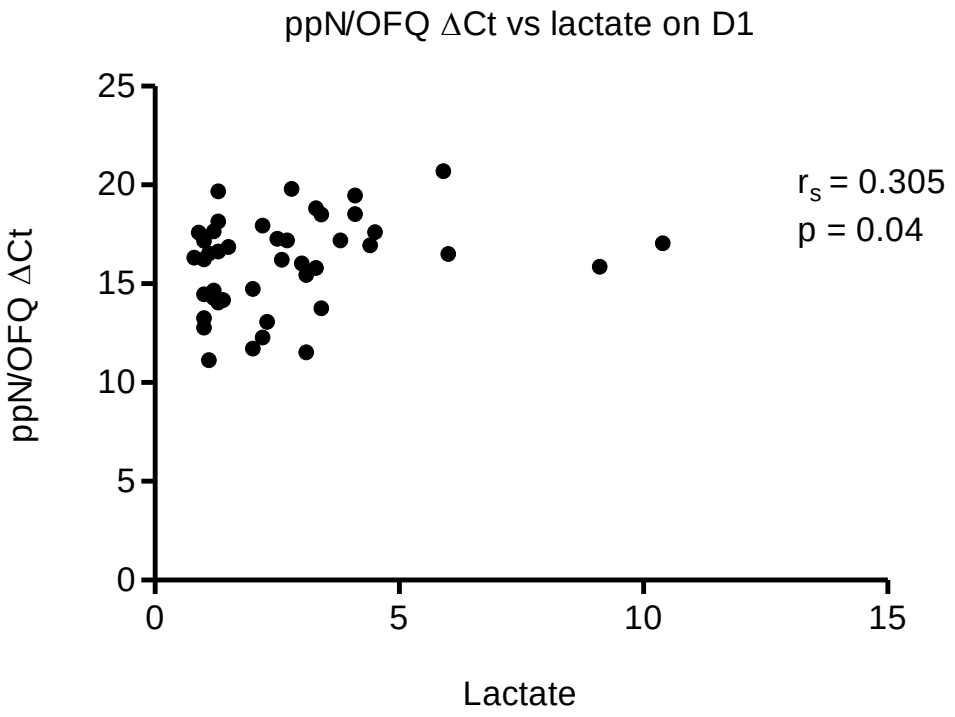


Figure 5-10 Correlation of Lactate vs ppN/OFQ ΔCt on Day 1 of sepsis. n=43, ppN/OFQ mRNA was undetermined in 6 samples. Data were analysed using non-parametric Spearman correlation (r_s)

5.4.9 Survivors and non-survivors NOP and ppN/OFQ mRNA expression

The mRNA expression of NOP and ppN/OFQ by polymorphonuclear cells was similar in those who died of sepsis compared to those who survived. **Figure 5-11**

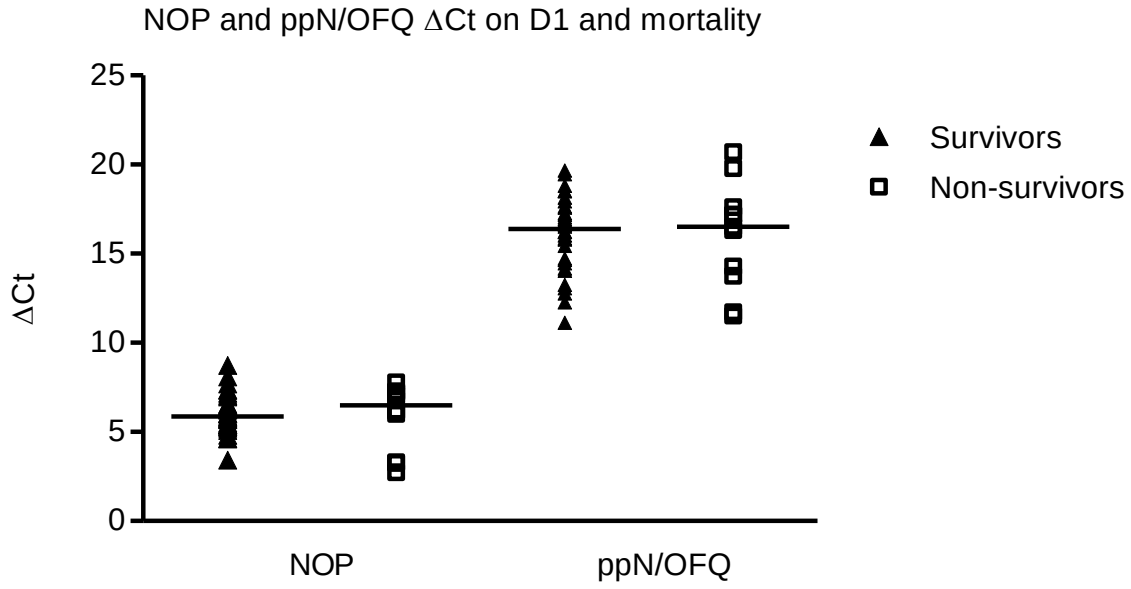


Figure 5-11 Correlation of NOP and ppN/OFQ ΔCt on D1 and mortality. NOP survivors n=36, non-survivors n=12. ppN/OFQ survivors n=32, non-survivors n=11. p= 0.43 and 0.98 for NOP and ppN/OFQ respectively. Data were analysed using Mann-Whitney U test.

5.4.10 Correlation between clinical findings and changes in mRNA expression of NOP and ppN/OFQ in the recovery sub-group, n=22

We analysed the correlation between various clinical findings and the change in mRNA expression of NOP and ppN/OFQ in Day 1 of sepsis compared to Recovery sample as the baseline (ΔΔCt).

Table 5-9**.** Data shows that those patients with a higher lactate on D1 of sepsis had a lower mRNA expression of NOP, when compared to the recovery sample (given by a higher ΔΔCt, D1 ΔCt minus recovery ΔCt). **Figure 5-12**. A higher APACHE score on admission to ITU was related to a higher ppN/OFQ mRNA expression (lower ΔΔCt), when compared to the recovery sample. **Figure 5-13**.

	NOP ΔΔCt (n=22)		ppN/OFQ ΔΔCt (n=19)	
	r_s (95% CI)	p value	r_s	p value
Age	-0.064 (-0.48 to 0.37)	0.77	0.068 (-0.41 to 0.51)	0.78
Sex	0.143 (-0.30 to 0.54)	0.52	-0.057 (-0.50 to 0.41)	0.81
Source of sepsis	-0.083 (-0.49 to 0.36)	0.71	0.104 (-0.37 to 0.54)	0.67
APACHE	0.310 (-0.14 to 0.65)	0.15	-0.629 (-0.84 to -0.23)	**0.003**
SOFA score	0.028 (-0.40 to 0.45)	0.9	-0.159 (-0.58 to 0.33)	0.51
Noradrenaline use	0.222 (-0.23 to 0.59)	0.31	0.224 (-0.26 to 0.62)	0.35
Creatinine	-0.003 (-0.43 to 0.42)	0.98	0.038 (-0.43 to 0.49)	0.87
WCC	-0.195 (-0.57 to 0.25)	0.38	-0.269 (-0.65 to 0.22)	0.26
% Neutrophils	-0.395 (-0.70 to 0.04)	0.06	-0.035 (-0.49 to 0.43)	0.88
Lactate	0.502 (-0.08 to 0.76)	**0.01**	-0.163 (-0.58 to 0.32)	0.5
ITU stay days	0.036 (-0.40 to 0.46)	0.87	-0.186 (-0.60 to 0.30)	0.44

Table 5-9 Correlation between NOP and ppN/OFQ ΔΔCt (D1 ΔCt minus Rec ΔCt) with demographic and physiological variables. ppN/OFQ ΔΔCt was undetermined in 3 patients. Data were analysed using non-parametric Spearman correlation (r_s). 11 variables were correlated. CI, confidence interval.

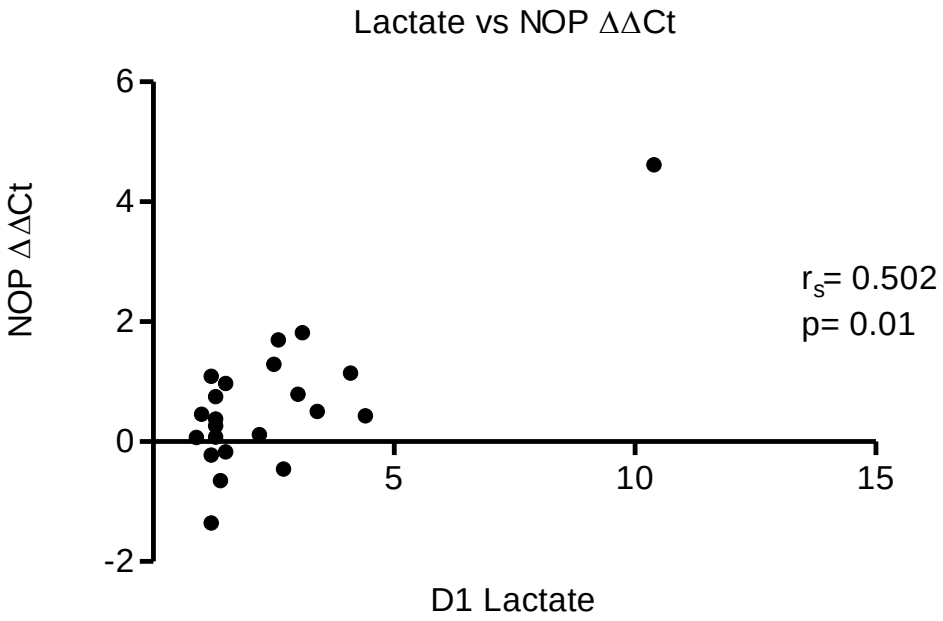


Figure 5-12 Correlation of Lactate on D1 vs NOP ΔΔCt (difference between D1 ΔCt minus recovery ΔCt), n=22. Data were analysed using non-parametric Spearman correlation (r_s).

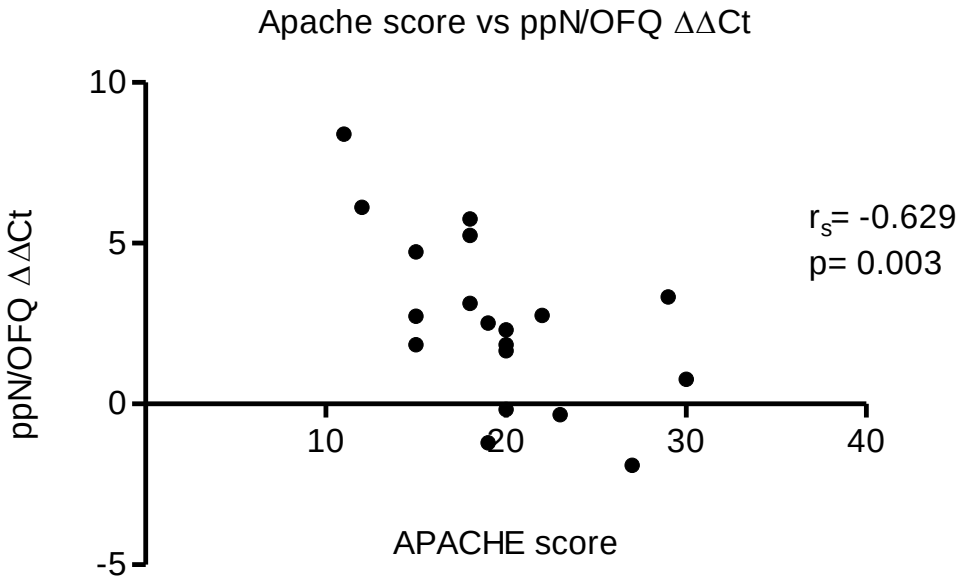


Figure 5-13 Correlation of APACHE score on admission to ITU vs ppN/OFQ ΔΔCt (D1 ΔCt minus Recovery ΔCt). n=19. ΔΔCt was undetermined in 3 patients. Data were analysed using non-parametric Spearman correlation (r_s).

5.4.11 Inflammatory markers – Clinical and biochemical

Clinical and biochemical markers of sepsis were investigated during sepsis and on recovery from sepsis. IL-8, IL-10, CRP and SOFA score increased significantly on Day 1 of sepsis compared to non-sepsis (Recovery), confirming the diagnosis of sepsis. TNFα plasma concentrations during sepsis were similar to those reported in the literature for patients with sepsis,[129] even though it did not increase significantly on Day 1 or Day 2 of sepsis compared to recovery. **Table 5-10**.

	Recovery	**D1**	**D2**
IL-8	41 (23 – 280) n=22	378 (179 – 1149)* n=49	297 (68 – 852)* n=46
IL-10	29 (3 – 250) n=22	161 (73 – 431)* n=49	118 (51 – 381) n=46
TNFα	33 (16 – 86) n=21	96 (43 – 168) n=49	66 (29 – 160) n=46
CRP	37(17 – 84) n=17	205 (128 – 277)* n=33	223 (164 – 284)* n=35
SOFA score	1 (0 – 1.2) n=18	6 (4.5 – 8.5)* n=49	5 (3 – 7)* n=46

Table 5-10 Inflammatory markers. Cytokine concentrations ($pg.ml^{-1}$), CRP ($mg.dl^{-1}$) and SOFA score. Data expressed as median (IQR). IL-8 and IL-10 concentrations were significantly higher during sepsis, *p value <0.01 compared to Recovery. Data were analysed using Kruskal-Wallis ANOVA with Dunn's multiple comparison test. SOFA: Sequential Organ Failure Assessment.

5.5 Discussion

These data showed that in critically ill patients with sepsis, ppN/OFQ mRNA expression by PMN cells is reduced by 85% during sepsis compared to recovery from sepsis. However, there were no changes in the mRNA expression of NOP. A similar finding was reported by Stamer and colleagues,[34] who investigated 18 critically ill patients with severe sepsis, and compared them to 28 healthy volunteers as the control group. They reported reduced mRNA expression of ppN/OFQ in pheripheral blood cells, and in contrast to our findings they observed increased mRNA expression of NOP in patients with sepsis compared to healthy controls.

Figure 5-14 .

The control group in our study was the patient itself. The recovery sample was taken when the patient had clinically recovered from sepsis. Despite the advantages that this has by providing a sample from the patient with same physiological and clinical background (opposed to volunteer controls), it has the problem of the timing of the sample. We obtained the recovery sample at a variable time between 7 and 134 days post recruitment, at home or in hospital, when patients were judged to have recovered from the episode of sepsis, based on clinical evaluation. However, this clinical evaluation did not necessarily correlate with complete physiological recovery, and full recovery from critical illness may take months. There are no data available on the time the nociceptin system specifically can take to return to baseline values (i.e. before sepsis)

The findings from this study do not support our hypothesis; if PMN cells are the source of plasma N/OFQ during sepsis, the mRNA expression of NOP and ppN/OFQ

would be higher. However, it shows that there is a change in mRNA expression of ppN/OFQ in the PMN cell during sepsis and it is possible that ppN/OFQ is used for making more active peptide and therefore its expression reduces.

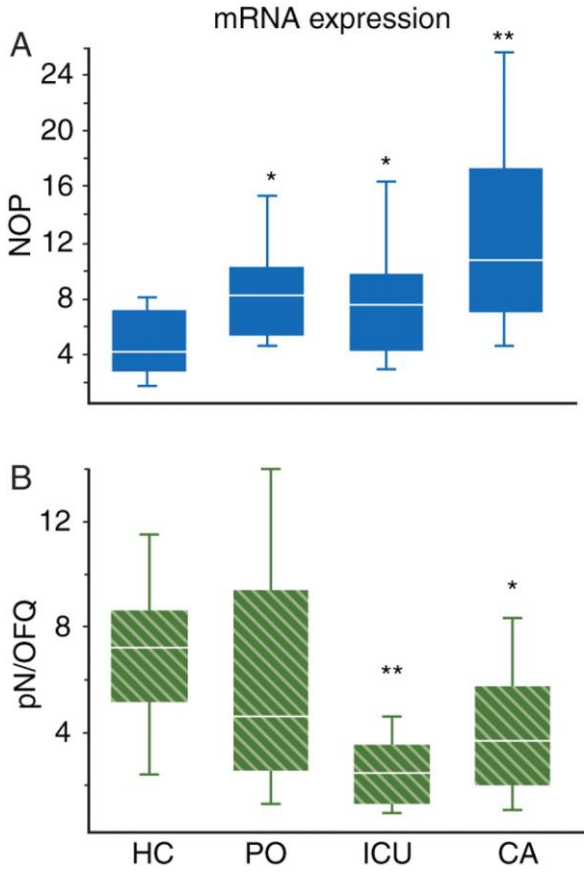


Figure 5-14 NOP and ppN/OFQ mRNA expression by peripheral blood cells in Stamer and colleagues' study.[34] mRNA expression is presented as normalized ratio. Graph A shows NOP mRNA expression was increased in ICU patients with sepsis (those who died of sepsis (n=9), not in survivors) compared to healthy controls (HC). Graph B shows ppN/OFQ mRNA expression was decreased in ICU patients with sepsis compared to healthy controls (HC). Stamer and colleagues also investigated cancer patients (CA) and post-operative patients (PO).
Graph taken from http://bja.oxfordjournals.org/content/106/4/566.long#F1. Accessed on 6th September 2012.

We found no difference in NOP or ppN/OFQ mRNA expression by PMN cells between survivors and non-survivors (36 and 13 patients respectively) of sepsis in 49 critically ill patients. However, we observed that patients who required higher doses of noradrenaline and those who had higher plasma lactate concentrations had reduced ppN/OFQ mRNA expression. Similarly, Stamer and colleagues observed reduced ppN/OFQ mRNA expression in those patients with higher concentrations of inflammatory markers, procalcitonin and IL-6. This evidence suggests that ppN/OFQ mRNA expression by leucocytes correlates with severity of clinical condition (increased noradrenaline requirements, increased plasma lactate, procalcitonin and IL-6 concentratios).

Mortality in critically ill patients with sepsis correlated with plasma N/OFQ concentrations and with NOP mRNA expression in Williams and colleagues and in Stamer and colleagues studies respectively. Williams and colleagues[33] studied 21 patients with sepsis and found significantly increased plasma concentrations of N/OFQ in 4 patients who died compared to survivors. **Figure 5-15**. Stamer and colleagues[34] studied 18 critically ill patients with severe sepsis and found higher NOP mRNA in peripheral blood cells of 9 patients who died compared to healthy controls. It is worth noting that Williams and colleagues compared survivors vs non-survivors, whilst Stamer and colleagues, compared patients to healthy volunteers.

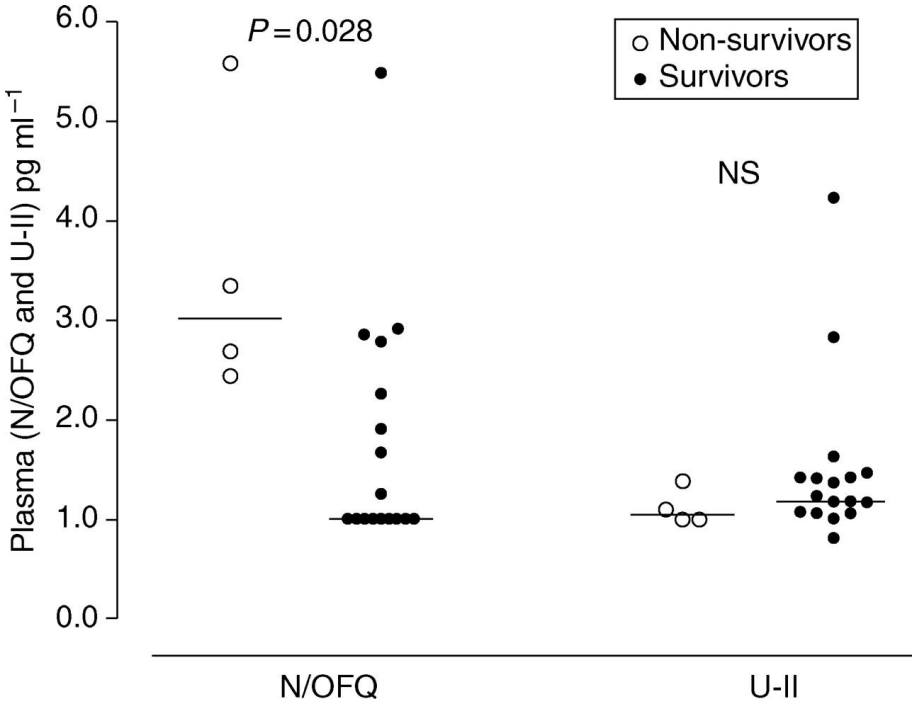


Figure 5-15 Plasma N/OFQ concentrations in critically ill patients with sepsis comparing survivors (n=17) and non-survivors (n=4), in Williams and colleagues study. Bar represents data medians. They also investigated Urotensin-II concentrations (U-II). Taken from http://bja.oxfordjournals.org/content/100/6/810.long Accessed on 8th September 2012.

During the first five days of sepsis, mRNA expression of NOP and ppN/OFQ by leucocytes and plasma concentrations of N/OFQ do not change significantly, as seen in this study as well as in the studies of Williams and colleagues[33] and Stamer and colleagues.[34] Plasma concentrations of sepsis biomarkers, such as CRP, IL-6, TNFα, and PCT increase or decrease during the first days of sepsis depending on the patient's clinical condition.[132;133] Sepsis evolves over a period of time that varies amongst patients. When the patient is admitted into the Intensive Care Unit the time course of the disease and the phase of the sepsis syndrome at which each patient presents is unknown. This variability contributes to admission delay, 'lead-time', and

is seen in the lack of significant clinical and laboratory changes between days 1 and 2 of sepsis. This is an inherent problem in most studies of sepsis along with accurate diagnosis.

Immunocytes play a key role in the development and outcome of the inflammatory response. We now know that immune cells such as polymorphonuclear cells, lymphocytes and monocytes express mRNA for NOP and ppN/OFQ. In the case of ppN/OFQ it is uncertain how much of this mRNA is translated into functional protein and released from the immunocyte.

5.5.1 Limitations of this study:

There are some limitations to these data:

1. All PCR studies are limited to investigating the mRNA expression of a given gene; however, this does not necessarily translate into the amount of protein, which is the critical response to analyse.
2. We have chosen to analyse PMN cells as this cell group is more likely to increase during a septic process. However, it would be worth studying the response of lymphocytes and monocytes as well. The method by which PMN cells were isolated provides over 90% cell purity. In future studies the use of FACS will allow for nearly 100% cell purity and analysis of mRNA in all white cell sub-types.
3. We have recruited patients with a clinical diagnosis of sepsis in their first 24 hours of ICU admission. However, this does not represent the first 24 hours of sepsis, as the time course in an individual patient varies and we do not know

the actual start time of sepsis, there is an admission delay (lead-time) into the ICU.[134]

4. We have compared the mRNA expression of the N/OFQ system using the patient itself as their own control. Further analysis should be performed using matched healthy controls as well.

5.6 Conclusion

Sepsis is a severe inflammatory response that occurs in the presence of infection and triggers multiple inflammatory pathways. It leads to multiple organ failure and causes death in 35% of patients admitted with sepsis to Intensive Care Unit. The N/OFQ system has been implicated in the adverse outcome from sepsis in animal studies. In human studies plasma N/OFQ concentrations were increased in patients who died from sepsis compared to survivors.

This observational, pilot study involving 49 patients with sepsis in Intensive Care, showed that mRNA expression of the N/OFQ system (NOP and ppN/OFQ) by polymorphonuclear cells, is similar during the first two days of sepsis, however, ppN/OFQ mRNA expression is reduced 85% during sepsis compared with non-sepsis status in the same patients (n=22).

Higher requirements for noradrenaline and higher lactate concentrations were associated with lower ppN/OFQ mRNA expression by PMN cells on the first day of sepsis.

To date, this is the second study available in the literature, to examine gene expression of the N/OFQ system by immune cells in humans with sepsis. Our research group will further compare and analyse these data with matched healthy volunteers.

6 Discussion

Previous chapters in this thesis have described the pharmacology of N/OFQ and an evaluation of its role in two clinical models of inflammation (i) CPB and (ii) sepsis. Based on animal studies, it is known that in pain pathways N/OFQ produces analgesia when administered at spinal sites and hyperalgesia/anti-opioid actions when administered supraspinally.[18] In humans, the evidence from observational studies shows both increased plasma N/OFQ concentrations in acute and chronic pain, and decreased plasma N/OFQ concentrations in fibromyalgia syndrome patients. N/OFQ has anxiolytic effects while NOP antagonists are antidepressants (animal studies). One human observational study showed plasma N/OFQ concentrations to be increased in patients with post-partum depression compared to healthy controls.

Cardiovascular effects of N/OFQ in animals include hypotension, vasodilatation, and bradycardia. In humans, NOP partial agonist SER100 (previously known as ZP120[135]) completed a Phase II trial for the management of decompensated heart failure in patients treated with furosemide, having as end-point a change in dyspnoea severity. Basic information about this un-published trial, sponsored by Serodus (previously Zealand Pharma), can be found on http://clinicaltrials.gov/ct2/show/ NCT00283361.

Our main interest on N/OFQ was regarding its effect on the immune system and its role during inflammation and sepsis. It is known that N/OFQ causes an inflammatory response, and increased mortality in septic rats, but more importantly, that UFP-101, a NOP antagonist, reduced mortality in septic animals. These actions were

demonstrated in two animal studies. *In vitro* N/OFQ caused increased macromolecular leak, vasodilatation, decreased blood flow and leucocyte rolling and adhesion in rat mesenteric vessels.[21] *In vivo* rats made septic by CLP (caecal ligation and perforation) had a mortality of 70%, which increased to 100% with sc administration of N/OFQ, and reduced to 50% with UFP-101, a NOP antagonist.[31] In the latter study, a decreased number of bacteria in the blood was detected in the rats treated with UFP-101, but it is unknown whether this is a direct or indirect effect of UFP-101.[136] This significant reduction in mortality of septic animals makes us wonder if a similar effect could be obtained in patients with sepsis.

Kimura and colleagues[137] explain a possible mechanism by which N/OFQ may contribute to the inflammatory process. They observed increased vascular permeability in rat skin regulated by histamine release, as it was antagonized by the anti-histamine pyrilamine. This effect had a very short onset, 30 seconds after sc injection of N/OFQ. They also observed that N/OFQ stimulated histamine release from rat peritoneal mast cells in a dose-dependent manner.

Two observational studies in critically ill patients with sepsis suggest that high plasma concentrations of N/OFQ correlate with patients who die of sepsis compared to survivors,[33] and that NOP and ppN/OFQ mRNA expression by leucocytes is altered during sepsis.[34]

In vitro studies confirm that PBMCs and PMN cells express ppN/OFQ and NOP mRNA.[9;14-17;23] It is known that toxin-activated human PMN cells and lymphocytes express N/OFQ, and that N/OFQ has pro- and anti-inflammatory effects on immunocytes. **Figure 6-1**

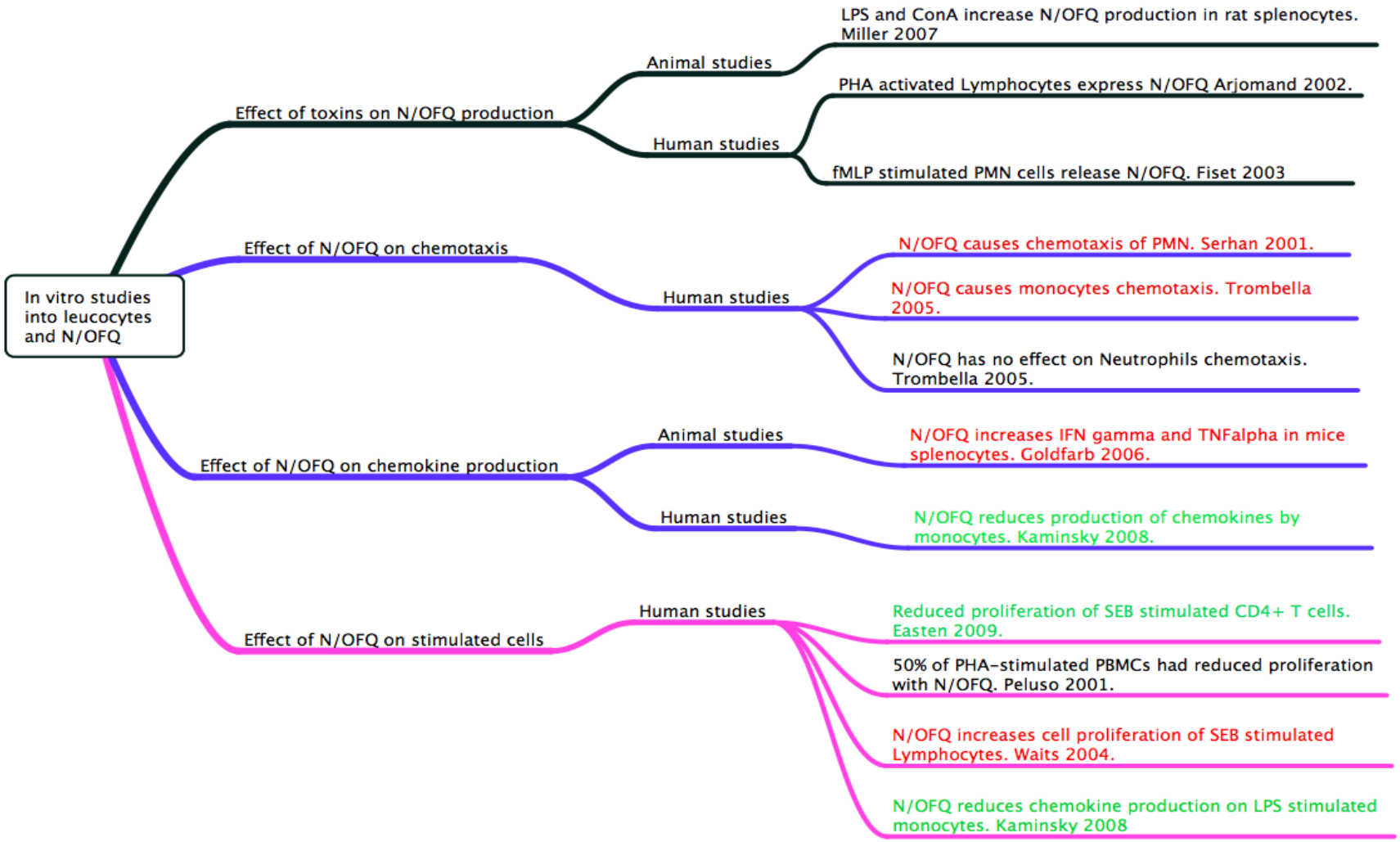

In vitro studies into leucocytes and N/OFQ
Effect of toxins on N/OFQ production
Animal studies
LPS and ConA increase N/OFQ production in rat splenocytes. Miller 2007
Human studies
PHA activated Lymphocytes express N/OFQ Arjomand 2002.
fMLP stimulated PMN cells release N/OFQ. Fiset 2003
Effect of N/OFQ on chemotaxis
Human studies
N/OFQ causes chemotaxis of PMN. Serhan 2001.
N/OFQ causes monocytes chemotaxis. Trombella 2005.
N/OFQ has no effect on Neutrophils chemotaxis. Trombella 2005.
Effect of N/OFQ on chemokine production
Animal studies
N/OFQ increases IFN gamma and TNFalpha in mice splenocytes. Goldfarb 2006.
Human studies
N/OFQ reduces production of chemokines by monocytes. Kaminsky 2008.
Effect of N/OFQ on stimulated cells
Human studies
Reduced proliferation of SEB stimulated CD4+ T cells. Easten 2009.
50% of PHA-stimulated PBMCs had reduced proliferation with N/OFQ. Peluso 2001.
N/OFQ increases cell proliferation of SEB stimulated Lymphocytes. Waits 2004.
N/OFQ reduces chemokine production on LPS stimulated monocytes. Kaminsky 2008

Figure 6-1*In vitro* studies on the effects of N/OFQ on leucocytes and toxins on N/OFQ release. The diagram shows evidence for the effects of toxins on N/OFQ production, N/OFQ on chemotaxis, N/OFQ on chemokine production and effects of N/OFQ on stimulated cells. In red are those studies showing pro-inflammatory effects; in green those showing anti-inflammatory effect of N/OFQ; and in black those studies with no definite pro or anti-inflammatory effect and those analysing N/OFQ response.

However, the evidence in humans is limited: we do not know how the N/OFQ system changes during inflammatory and septic conditions and if there is any relationship with severity of disease.

Based on current available data the aims of the studies described in this thesis were to analyze changes in the expression of NOP and ppN/OFQ mRNA by PMN cells and of N/OFQ peptide in plasma during inflammation and sepsis. A further aim was to investigate the relationship between the N/OFQ system with physiological and biochemical indicators of severity of disease.

The work presented in this thesis represents the first investigations into: i) the changes of mRNA expression of NOP and ppN/OFQ by PMN cells specifically, as these cells play a key role in the development of the inflammatory response; and ii) the change of the N/OFQ system in plasma and in PMN cells, having the patient itself as his own control.

The purpose of this section is to discuss and interpret the findings of the studies described in Chapters 3, 4 and 5, and to outline possible future areas of research.

The response of the N/OFQ system during inflammation and sepsis by polymorphonuclear leucocytes was investigated in two different patient populations:

40 patients undergoing cardiac surgery under cardiopulmonary bypass (CPB) as a model of inflammation, and 49 patients with severe sepsis in the Intensive Care Unit. Recruitment of this number of patients took 18 and 21 months respectively and was particularly challenging in the ICU environment.

6.1 The effect of cardiopulmonary bypass on N/OFQ

The data presented in Chapter 3 showed that NOP and ppN/OFQ mRNA expression by PMN cells was significantly reduced at 3h post-CPB by 57% and 95% respectively. **Table 6-1** .

We investigated changes in the N/OFQ system in humans during inflammation, and chose patients undergoing cardiac surgery under cardiopulmonary bypass as a model of inflammation.

CPB is known to cause a systemic inflammatory response, which has been demonstrated by various authors by an increased plasma concentration of cytokines. Onorati and colleagues studied 60 patients and found increased plasma concentrations of IL-6, IL-8, and IL-10.[99] Duggan and colleagues found increased TNFα and IL-10 in 82 patients undergoing cardiac surgery under CPB.[112] Galley and colleagues reported increased plasma IL-10 concentrations in 150 patients.[111]

Other findings of the inflammatory response seen during CPB include endothelial cell activation; activation of complement, kinin, fibrinolytic and coagulation cascades; and activation and aggregation of neutrophils.[100] PMN cells are activated by contact of the cell with the CPB circuit, and this activation is enhanced by raised cytokines. Activated PMN cells release proteolytic enzymes: MPO (myeloperoxidase), MMPs (matrix-metalloproteinases), PMN elastases and oxygen-free radicals, all of which promote organ dysfunction.[88;89]

The above pathophysiological changes lead to deleterious clinical effects such as atrial fibrillation,[138] lung dysfunction,[139] renal impairment, gastrointestinal and metabolic changes, cognitive dysfunction,[140] and stroke.[115;141]

Some of the mechanisms that lead to the inflammatory response during CPB include contact of blood with foreign surfaces in the extracorporeal circuit, non-physiologic pulsatile perfusion and ischemia-reperfusion injury due to cardioplegic arrest.[99]

In our study, inflammatory markers IL-8, IL-10 and MPO significantly increased with the inflammatory response. IL-8 increased 7-fold at 3h post-CPB, IL-10 4-fold, and MPO increased by 63%. Plasma N/OFQ concentration increased directly in proportion to the increase of MPO.

Sablotzki and colleagues,[142] investigated the relationship between inflammatory markers and mortality in 16 post-CPB patients with multi-organ dysfunction syndrome and APACHE-II score >24, and reported increased plasma concentrations of IL-8, IL-10, IL-18, TGF (transforming growth factor), and procalcitonin in those who died compared to survivors.

Plasma TNFα concentrations did not change in our study. However, other studies have reported an increase of TNFα and associated it with clinical findings. Abacilar and colleagues studied 20 patients undergoing elective coronary artery bypass grafting (CABG) and correlated an increase of TNFα of more than 20 $pg.ml^{-1}$ in 13 patients with increased mediastinal bleed and longer endotracheal intubation.[143]

There is no evidence in the literature regarding mRNA expression of NOP or ppN/OFQ by leucocytes during inflammation. However, there is some evidence regarding G protein-coupled receptor kinase activity and NOP mRNA expression in human right atrium. Hagen and colleagues[144] investigated 15 patients undergoing cardiac surgery under CPB, and observed that G protein-coupled receptor kinase

activity was reduced in mononuclear cells, and that this change correlated with increased plasma concentrations of IL-6. Our group reported the presence of NOP mRNA in human right atrium of 38 patients undergoing cardiac surgery under CPB. We investigated 40 patients undergoing cardiac surgery under CPB and found reduced mRNA expression of NOP, a G protein-coupled receptor, in PMN cells.

Plasma N/OFQ concentrations in patients with coronary artery disease have been investigated by Fontana and colleagues, and by Krepuska and colleagues, reporting opposite findings. Fontana and colleagues reported increased plasma N/OFQ concentration in 41 patients with unstable angina compared to 7 patients with stable angina and to 20 controls. Krepuska and colleagues reported reduced plasma N/OFQ concentration in 12 patients with severe chronic angina compared to 10 patients with mild angina and to 14 healthy controls. Our results showed that plasma N/OFQ concentration increased by 31% at 3h post-CPB and returned to above baseline concentration at 24h post-CPB. High concentration of plasma N/OFQ was associated with low NOP mRNA expression by PMN cells. Prolonged aortic cross-clamp time was associated with higher concentration of plasma N/OFQ and lower NOP mRNA expression.

In an attempt to determine whether the reduction in mRNA expression of NOP and of ppN/OFQ by PMN cells during CPB was due to changes in plasma N/OFQ concentrations, we performed an *in vitro* study. We incubated extracted PMN cells, from the t0 sample of 4 patients, with 1 µM N/OFQ and peptidase inhibitor cocktail for 3h. We compared the results to control samples, PMN cells incubated on peptidase inhibitor only. We observed reduced expression of NOP and ppN/OFQ

mRNA after 3h of incubation on N/OFQ. However, the control samples, with only peptidase inhibitor (PI), had similar mRNA expression response as the N/OFQ samples. It is possible that PMN cells release N/OFQ into plasma during an inflammatory process, and peptidase inhibitor prevented the breakdown of the added and the intrinsic N/OFQ, therefore showing similar response in both groups. This was a very small number of patient samples analysed and deserves further investigation.

6.1.1 Unexpected findings in the CPB study

We hypothesized that an inflammatory stimulus such as cardio pulmonary bypass would cause an increase in N/OFQ plasma concentrations. Our research group had previously demonstrated that plasma N/OFQ concentrations increase in critically ill patients with sepsis.

With the evidence that NOP and ppN/OFQ mRNA is expressed by immunocytes,[9] we also hypothesized that these cells were the source of N/OFQ and therefore NOP and ppN/OFQ mRNA expression would be increased in PMN cells.

The CPB study showed an increase in plasma N/OFQ concentrations but a reduction in NOP and ppN/OFQ mRNA expression by the polymorphonuclear cell, opposite of our hypothesis. We propose two possible mechanisms for this response: 1) when plasma N/OFQ concentrations increase in response to the inflammatory process, ppN/OFQ and NOP mRNA expression fall as they are degraded to produce protein, and/or 2) immunocytes release N/OFQ during the inflammatory process and this leads to a negative feedback, reducing the expression of NOP and ppN/OFQ from the immune cells.

We measured inflammatory markers IL-8, IL-10, TNFα and MPO. TNFα was the only inflammatory marker that failed to increase at 3 hours after CPB compared to baseline values. Other CPB studies have shown increased TNFα response as part of the inflammatory response.[114;117;139] The reasons why we observed an increase in IL-8, IL-10 and MPO, but not in TNFα, in response to CPB are unclear but may relate to differences in techniques used for CPB between studies.

We also observed that 4 patients had ten times higher plasma concentrations of IL-8, IL-10 and TNFα. However, there was neither clinical nor physiological explanation for this finding and no change in outcome compared to the other 36 patients.

6.2 The effects of sepsis on N/OFQ

Sepsis has a complex pathogenesis involving a sequence of cellular processes such as activation of neutrophils and monocytes; microvascular endothelial cells; and activation of the complement, coagulation and fibrinolytic cascades. These are necessary processes in order to clear the body of pathogens. However, there is a cascade of mechanisms triggered by pathogen-derived signals leading to damage-associated molecular pattern (DAMP) molecules being released from epithelial and endothelial cells that maintain the inflammatory mediators in a positive feedback mechanism. Excessive pro-inflammatory or anti-inflammatory mediators, which can lead to an immunosuppressant state and opportunistic secondary infections, that persist even after the pathogen has been cleared, are the basis of sepsis and multi-organ failure.[74;75]

Most commonly used inflammatory markers during sepsis include C-reactive protein (CRP) and procalcitonin (PCT). Beyond these molecules a wide range of biomarkers

have been investigated, including cytokines (IL-6, IL-8, IL-10, TNFα),[127;129] coagulation factors, and vasoactive hormones.

N/OFQ has been linked to sepsis based on *in vitro*, and *in vivo* animal and human studies described at the beginning of this chapter.

We investigated the N/OFQ system in 49 critically ill patients with sepsis during their first 48h in the Intensive Care Unit. In 22/49 patients a blood sample was also obtained once the patient had recovered from sepsis, and this was often taken at home after discharge. NOP and ppN/OFQ mRNA expression by PMN leucocytes was similar on Days 1 and 2 of sepsis. However, compared to recovery from sepsis, ppN/OFQ mRNA expression decreased 85% during sepsis. **Table 6-1** .

In a very recent study Stamer and colleagues,[34] investigated 18 critically ill patients with severe sepsis, and compared them to 28 healthy volunteers as the control group, also reported reduced mRNA expression of ppN/OFQ in peripheral blood cells. It is important to note that we investigated the PMN cell specifically whilst Stamer and colleagues investigated peripheral blood cells (mononuclear and polymorphonuclear cells).

Stamer and colleagues reported increased mRNA expression of NOP in patients with sepsis compared to healthy controls. This contrasts our findings, where there was no difference in the mRNA expression of NOP on Day 1 of sepsis compared to recovery from sepsis.

When correlating NOP and ppN/OFQ mRNA expression with clinical and laboratory findings, we observed that a higher APACHE score on admission to ITU was related to a higher ppN/OFQ mRNA expression (lower $\Delta\Delta$Ct), when compared to the recovery sample. On day 1 of sepsis the need for higher doses of positive inotropic drugs and higher serum lactate concentrations were both associated with lower

ppN/OFQ mRNA expression. These are the first correlations reported between the N/OFQ system and indicators of severity of disease in patients with sepsis.

Williams and colleagues previously investigated 21 patients with sepsis and reported increased plasma N/OFQ concentrations in 4 patients who died of sepsis compared to survivors. In view of the presence of NOP and ppN/OFQ mRNA in PBMC and PMN cell, it is possible that the origin of plasma N/OFQ is the immune cell and therefore its mRNA expression is altered during sepsis. Stamer and colleagues[34] reported higher NOP mRNA expression in peripheral blood cells of 9 patients who died of severe sepsis compared to healthy controls. We did not find any difference in the mRNA expression of NOP or of ppN/OFQ when comparing survivors (n=36) and non-survivors (n=13) from sepsis.

6.2.1 Unexpected findings in the sepsis study

Our data showed an 85% reduction in ppN/OFQ mRNA expression by PMN cells during sepsis with no change in NOP mRNA expression. These findings differ from our hypothesis that if PMN cells are the source of plasma N/OFQ during sepsis its mRNA expression of NOP and ppN/OFQ would be higher.

The reduced ppN/OFQ mRNA expression is similar to the changes observed in the CPB study, where we believe this may be due to either ppN/OFQ being degraded to produce protein, or to a negative feedback from higher concentrations of N/OFQ peptide.

In contrast to the reduced expression of ppN/OFQ mRNA, the expression by PMN cells of NOP mRNA did not change during sepsis when compared to recovery from sepsis..

The lack of differences in the NOFQ system between days 1 and 2 of admission to ICU may relate to heterogeneity in disease processes, illness severity and responses to resuscitation or treatment. Alternatively there is a recognised 'lead-time' before ICU admission and the disease process for an individual will have evolved over a number of hours or days before they present to hospital or are admitted to ICU. In this context, repeated sampling after 24 hours may be too short an interval to demonstrate marked differences in the complex interacting pathways involved in sepsis.

	mRNA expression		Plasma	IL-8	IL-10	TNFα	MPO	Correlations	
	NOP	ppN/OFQ	N/OFQ						
CPB study	↓ 57%	↓ 95%	↑ 31%	↑ 7-fold	↑ 4-fold	NC	↑ 63%	Longer AXC time	↓ NOP mRNA ↑ plasma N/OFQ
								↑ plasma N/OFQ	↓ NOP mRNA
Sepsis study	NC	↓ 85%	ND	↑ 9-fold	↑ 5-fold	NC	ND	↑ inotropic support	↓ ppN/OFQ mRNA
								↑ lactate	↓ ppN/OFQ mRNA

Table 6-1 Summary of findings of cardiopulmonary bypass (CPB) and sepsis studies. NC, no significant change. ND, not done.

6.3 Relevance of the N/OFQ system in inflammation and sepsis

Both N/OFQ and NOP are involved in a wide range of 'non-pain' responses including immunomodulation and cardiovascular control. There is growing interest in the involvement of this system during inflammation and sepsis.

The N/OFQ - NOP system is present in immune cells and N/OFQ modifies immunocyte function. NOP and N/OFQ precursor ppN/OFQ mRNA are found in monocytes, lymphocytes and polymorphonuclear cells.[9;14;15] *In vitro* studies show N/OFQ to be produced by immunocytes[29] and to act as an immunomodulator (N/OFQ causes chemotaxis of monocytes, PMN cells and increased cell proliferation on lymphocytes).[25;26] Its effects are mainly pro-inflammatory, e.g. induction of chemotaxis and proliferation of immune cells.

In vivo animal studies have been conducted in septic and non-septic animal models of inflammation. Pro-inflammatory effects were seen on mesenteric vessels of anaesthetised rats when N/OFQ was injected iv: leucocyte rolling & adhesion, increased macromolecular leak, reduced blood flow, and vasodilatation.[21] Increased mortality was seen on septic CLP rats after sc injection of N/OFQ. Furthermore, there was reduced mortality after sc injection of the N/OFQ antagonist UFP-101 in the same study conducted by Carvalho and colleagues.[31] Opposed to these pro-inflammatory effects, an anti-inflammatory response was observed by Zhao and colleagues[32] when N/OFQ was injected centrally, intracerebroventricular, in anaesthetised rats. They studied rats undergoing exploratory laparotomy as a model of traumatic stress, and determined IL-1 and TNFα production by peritoneal macrophages in response to intracerebroventricular injection of N/OFQ. They observed increased production of IL-1 and TNFα from peritoneal macrophage in

response to the traumatic stress, and this was reversed with intracerebroventricular injection of N/OFQ. They also observed that N/OFQ and NOP mRNA transcripts were reduced in cerebral cortex, hippocampus and hypothalamus after trauma, and this could be reversed by central administration of N/OFQ. The opposite response to centrally and peripherally administered N/OFQ is also seen in response to pain.

In humans, two observational studies in critically ill patients with sepsis have been published. One study showed raised plasma N/OFQ concentrations in patients who died of sepsis compared to survivors,[33] and the other study showed increased NOP mRNA expression by peripheral blood cells in those patients who died, with reduced ppN/OFQ mRNA expression in patients with sepsis compared to controls.[34] Limited data are available regarding the N/OFQ system in patients with SIRS.

6.4 Problems encountered during the studies

6.4.1 CPB study

We faced the following problems recruiting patients into the CPB study:

a) cancellations of surgery on the day due to lack of ITU beds for the post-operative period,

b) low volume of surgical cases during a period of time due to bed closures,

c) some patients denied consent, and

d) we were limited to recruiting patients whose surgery started before midday as samples took 2 – 3hours to process in the laboratory.

Regarding timing of the second blood sample, we took the t2 sample 3 hours after start of CPB (instead of after the end of CPB). In 33/40 patients CPB time was 1 – 2 hours, in 3 patients we took the blood sample whilst the patient was still on CPB

(CPB time of 203, 204 and 250 minutes), one of these patients was clinically unstable requiring CPB a second time. One patient had a short CPB time of 43 minutes. We were consistent on sampling all patients at the same time in relation to when they were started on CPB, the inflammatory stimulus.

6.4.2 Sepsis study

During the sepsis study 2 patients were withdrawn from the study. One after the first sample was taken assent was denied by the relatives, the second patient had relatives under age in which case assent was unacceptable. In both cases any blood samples and records collected were destroyed.

We took a recovery blood sample once the patient had recovered clinically from sepsis. This timing varied amongst patients, some were at hospital, and others were at home. The recovery sample was obtained in 22/49 cases, 13 patients died of sepsis within 30 days, and 2 patients died after 30 days of ICU admission. Reasons for not been able to obtain the recovery samples in the other patients included: transfer to another hospital, denial of consent for the recovery sample, unable to contact the patients, and some lived over 30 minutes away from the hospital so we would have been unable to process the blood samples within an acceptable time.

We used the patient themselves to act as their own control, to account for the effects of co-morbidities, age, medication and other unknown factors that might affect the N/OFQ system. Ideally one would like to take the control sample before the insult occurs, however this is clearly impossible to do in with patients presenting as an emergency with sepsis.

An alternative would be to obtain reference samples from healthy volunteers matched by age and sex to the study group. This has the advantage that we could

match all of the study patients, including those who died of sepsis. This might exclude some potential confounding variability and is an option for further research.

6.5 Suggestions for future research

We investigated the expression of the N/OFQ system by PMN cells in patients exposed to an inflammatory process, and in critically ill patients with sepsis. To date, there are various *in vitro* and *in vivo* studies showing the pro-inflammatory effect of N/OFQ. However, there is limited information on the behaviour of the N/OFQ system in humans during inflammation and sepsis. Further clinical studies are needed to investigate the protein expression of N/OFQ by immunocytes; in particular each immune cell type.

6.6 Conclusion

The expression of the N/OFQ - NOP system by the PMN cell changes in patients exposed to an inflammatory process and to sepsis. Plasma concentration of N/OFQ increases during inflammation whilst NOP and ppN/OFQ mRNA expression by PMN cell decreases. Prolonged aortic cross clamp time during CPB is associated with higher N/OFQ plasma concentrations. High noradrenaline requirement and lactate concentrations during sepsis are associated with low NOP and ppN/OFQ mRNA expression by the PMN cell.

Changes in the mRNA expression of the N/OFQ receptor NOP, and the N/OFQ peptide precursor ppN/OFQ in the PMN cell along with changes in plasma N/OFQ concentration, suggest that the PMN cell is the source of plasma N/OFQ during inflammation and sepsis. These data support previous work which strongly suggest the involvement of the NOFQ system in inflammatory processes. Further work is

required to elucidate the precise role, and whether pharmacological manipulation of the N/OFQ system in sepsis would be useful in clinical practice.

7 Appendix

7.1 CPB Study: Ethics approval

NHS
National Research Ethics Service

Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1

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30 June 2008

Dr Simon P Young
Specialist Registrar & Honorary Lecturer
University Hospitals of Leicester NHS Trust
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Dear Dr Young,

Full title of study: **Role of the nociceptin system in the stress response - a study of patients undergoing cardiac surgery**

REC reference number: **08/H0406/103**

Thank you for your letter of 26 June 2008, responding to the Committee's request for further information on the above research and submitting revised documentation, subject to the conditions specified below.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements.

This Research Ethics Committee is an advisory committee to East Midlands Strategic Health Authority.
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England.

7.2 CPB study: Participant Information Sheet

PARTICIPANT INFORMATION SHEET v.3 (16 August 2009)

Study Title: Role of nociceptin in the stress response.

You are being invited to take part in a research study. Participation is entirely voluntary. Please take time to read the following information carefully and discuss it with others if you wish. If you agree to take part, we will ask you to sign a consent form. You are free to withdraw from the study at any time, without giving a reason; any blood samples you have donated will be destroyed.

Why have I been chosen?

Our laboratory has discovered an increase in blood levels of a 'hormone' called nociceptin when the body is under stress. We believe that this hormone may originate from the white cells in the blood. We wish to measure the production of nociceptin by these white blood cells in patients undergoing cardiac surgery. With the information gained from this study we ***may*** in the future be able to modify how this hormone works, and improve the way the body reacts to stress.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you agree to take part you can change your mind at any time and withdraw from the study without giving a reason. Whether you take part or not, the standard of care you receive will not be different in any way.

What will happen if I take part?

Your involvement in the study will only last as long as you are patient in hospital. We will take a total of three small blood samples from you during and after the operation. These will be taken from a special 'drip' (or 'tap') in your artery that will be placed anyway as part of your anaesthetic, whether or not you are involved in the study. This means that no extra needles will be used, and the taking of blood samples should be painless. The total amount of blood taken will be 90 ml, or approximately

half a tea cup-full. Once the blood samples have been taken, you will have no further involvement in the study. Your recovery after surgery will continue as it would normally.

What are the advantages & disadvantages of taking part?

The information we obtain from the study will not affect you directly, but may help us to improve the treatment that future patients receive. You will not receive any payment for the samples. Neither you nor your relatives will benefit financially from any inventions that result from the use of the blood samples.

What will happen to the results of the study?

Full analysis of the blood samples will take approximately 12 months. The blood samples will be frozen & stored in locked laboratory facilities in the Department of Anaesthesia, Critical Care & Pain Management, Leicester Royal Infirmary. Once the tests have been performed, any remaining blood samples will be destroyed. It is our intention to publish the results of the study in medical journals and at medical meetings. This may however take a number of years.

Is the study confidential?

All information collected about you for this study will be kept strictly confidential, and is locked away in offices & laboratories of the Department of Anaesthesia, Critical Care & Pain Management, Leicester Royal Infirmary. Any information from the study that is reported will **not** use your name or address or any other information that would allow you to be recognised.

What if I am harmed by the study?

We do not anticipate any risk to you when donating blood samples. However, you should know that if you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence then you may have grounds for a legal action, but you may have to pay for it yourself. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you. In the

University Hospitals Leicester NHS Trust there is a free & confidential service called the Patient Advice & Liaison Service (PALS). Their contact details are as follows:

PALS Office
Glenfield Hospital
Groby Road
Leicester
LE3 9QP
Tel: 0116 258 3100
e-mail: pals@uhl-tr.nhs.uk

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by the local Research Ethics Committee.

Contact for further information

Dr Alcira Serrano-Gomez (Principal Investigator)
Specialist Registrar & Honorary Lecturer
University Division of Anaesthesia, Critical Care & Pain Management
Department of Cardiovascular Sciences
3rd Floor, Victoria Building
Leicester Royal Infirmary
Leicester
LE1 5WW
Tel: 0116 258 5291 e-mail: asg21@le.ac.uk

Thank you for taking time to read this participant information leaflet.

7.3 CPB Study: Consent Form

Patient name, DOB and hospital number, or addressograph label

CONSENT FORM

Study Title: Role of nociceptin in the stress response.

Investigators: Dr A Serrano-Gomez, Dr J Thompson, Prof D Lambert

Patient to initialise boxes

1. I confirm that I have read and understood the participant information leaflet dated 16 August 2009, version 3, and have had the opportunity to ask questions. ☐

2. I understand that any blood samples are a gift and that I will not benefit from any intellectual property that results from their use. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, and without my medical care or legal rights being affected. ☐

4. I understand that sections of my medical records may be looked at by responsible individuals from the research team or from regulatory authorities. I give permission for these individuals to have access to my records. ☐

5. I hereby consent to donate blood samples (total 90 ml), and to have these samples stored in the laboratory for testing of 'stress' hormones including nociceptin/orphanin FQ and cytokines. My blood samples will be destroyed once these tests have been performed. ☐

Name of Patient _____

Signature of Patient _____ Date _____

Name of Researcher _____

Signature of Researcher_____Date _____

Original form to be held in University Office, copies to Patient and Hospital Notes

7.4 Sepsis Study: Ethics Approval

NHS
National Research Ethics Service

Camden & Islington Community Research Ethics Committee
REC Offices
South House, Royal Free Hospital
Pond Street, London
NW3 2QG

Telephone: 020 7794 0500 extn 36906
Facsimile: 020 7794 1004

27 March 2009

Dr Alcira Serrano-Gomez
Division of Anaesthesia
3rd Floor, Victoria Building
Leicester Royal Infirmary
University Hospitals of Leicester NHS Trust
LE1 5WW

Dear Dr Serrano-Gomez

Full title of study: **Role of nociceptin in sepsis**

REC reference number: **09/H0722/21**

The Research Ethics Committee reviewed the above application at the meeting held on 23 March 2009. Thank you for attending to discuss the study. Apologies the Committee did not invite you into the meeting; it was not considered necessary as no further information was required.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Mental Capacity Act 2005

I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

7.5 Sepsis Study: QED Audit

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH & DEVELOPMENT
Director: **Professor D Rowbotham**
Acting Assistant Director: **Carolyn Maloney**

Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Direct Dial: (0116) 258 4199
Fax No: (0116) 258 4226

Tel: 0116 249 0490
Fax: 0116 258 4666
Minicom: 0116 258 8188

17th August 2009

Dr Alcira Serrano-Gomez
Division of Anaesthesia
Victoria Building
Leicester Royal Infirmary
Infirmary Square
Leicester
LE1 5WW

Dear Dr Alcira Serrano-Gomez

Re: **Role of nociceptin in sepsis**

UHL 10723

Thank you for helping QED to undertake an Audit for the above study on the 4th August 2009.

I enclose a copy of the findings from the Audit. QED found the study documentation to be of a good standard, and there are only minor findings to be addressed. I look forward to receiving a response to the findings as soon as possible, but in any event, by 14th September 2009.

Yours faithfully

Rebecca Pritchard
Clinical Trainer and Trials Monitor

Cc Carolyn Maloney

Encs.

7.6 Sepsis study: Participant Information Sheet

PARTICIPANT INFORMATION SHEET v.5 (25 January 2010)

Study Title: Role of nociceptin in sepsis.

Principal Investigator:

Dr Alcira Serrano-Gomez
Specialist Registrar & Honorary Lecturer
University Division of Anaesthesia, Critical Care & Pain Management
Department of Cardiovascular Sciences
3rd Floor, Victoria Building
Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 258 5291 e-mail: asg21@le.ac.uk

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.
Participation is entirely voluntary. If you agree to take part, we will ask you to sign a consent form to show that you agree to take part. You are free to withdraw from the study at any time, without giving a reason. If you withdraw, any blood samples you have donated will be destroyed.

What is the purpose of this study?

Our laboratory has discovered an increase in blood levels of a 'hormone' called nociceptin when the body is invaded by infection. We believe that this hormone may originate from the white cells in the blood. We wish to measure the production of nociceptin by these white cells in patients with infection. With the information gained from this study we ***may*** in the future be able to modify how this hormone works, and improve the way the body reacts to infection.

Why have I been invited?

You have been approached for this study because you have been admitted to the Intensive Care Unit at the Leicester Royal Infirmary with infection. We wish to measure the production of the 'hormone' nociceptin and other stress hormones by the white cells in the blood of patients having an infection. Our aim is to recruit 50 patients into this study.

Do I have to take part?

No, it is up to you to decide whether or not to take part.

What will happen if I take part?

Your involvement in the study will only last as long as you are patient in the hospital. We will take a total of three small blood samples from you, the first two in two consecutive days and the third one once you recover from the infection. The first two samples will be taken from a special cannula (or 'drip) in your artery or vein that will be placed anyway as part of your routine care in ICU, whether or not you are involved in the study. The third recovery sample may be taken with a needle from a vein, if the special cannulas have been removed. The total amount of blood taken will be 75 ml, or approximately half of a tea cup-full. The blood samples are not being DNA tested.

What are the advantages of taking part?

The information we obtain from the study will not affect you directly, but may help us to improve the treatment that future patients receive.

What are the possible disadvantages and risks of taking part?

We do not anticipate any risk to you when donating blood samples. There will be no impact on life insurance or private medical insurance.

What do I have to do after the study?

Once the blood samples have been taken, you will have no further involvement in the study. Your recovery after your stay in ICU will continue as it would normally.

Will I receive payment for the tissue I donate for this research study?

You will not receive any payment for the samples. The samples are a gift. Neither you nor your relatives will benefit financially from any inventions that result from the use of the blood samples.

What will happen to the results of the study?

Full analysis of the blood samples will take approximately 12 months. The blood samples will be frozen & stored in locked laboratory facilities in the Department of Anaesthesia, Critical Care & Pain Management, Leicester Royal Infirmary. Once the tests on your blood have been performed, any remaining blood samples will be destroyed. It is our intention to publish the results of the study in medical journals and at medical meetings. This may however take a number of years.

Is the study confidential?

All information collected about you for this study will be kept strictly confidential, and is locked away in offices & laboratories of the Department of Anaesthesia, Critical Care & Pain Management, Leicester Royal Infirmary. Any information from the study that is reported will **not** use your name or address or any other information that would allow you to be recognised.

What if I am harmed by the study?

We do not anticipate you being harmed by this study. However, you should know that if you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence then you may have grounds for a legal action, but you may have to pay for it yourself. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you. In the University Hospitals Leicester NHS Trust there is a free & confidential service called the Patient Advice & Liaison Service (PALS). Their contact details are as follows:

PALS Office
Glenfield Hospital
Groby Road
Leicester

LE3 9QP
Tel: 0116 258 3100
e-mail: pals@uhl-tr.nhs.uk

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by Camden & Islington Community Research Ethics Committee.

Who is organising and funding the research?

This study is being organised and funded by the University Department of Anaesthesia, Critical Care & Pain Management, based at Leicester Royal Infirmary and is being conducted as part of an educational award.

Thank you for taking time to read this participant information leaflet.

7.7 Sepsis study: Consent Form

Patient name, DOB and hospital number, or addressograph label

CONSENT FORM

Study Title: Role of nociceptin in sepsis.

Investigators: Dr A Serrano-Gomez, Dr M Wood, Dr N Ladak, Dr J Thompson, Prof D Lambert

Patient to initialise boxes

1. I confirm that I have read and understood the participant information leaflet dated 25 January 2010, version 5, and have had the opportunity to ask questions. ☐

2. I understand that any blood samples are a gift and that I will not benefit from any intellectual property that results from their use. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, and without my medical care or legal rights being affected. ☐

4. I understand that sections of my medical records may be looked at by responsible individuals from the research team or from regulatory authorities. I give permission for these individuals to have access to my records. ☐

5. I hereby consent to donate three blood samples (total 75 ml), and to have this sample stored in the laboratory for testing of 'stress' hormones (endogenous opioids, cytokines, endocannabinoids) and their receptors. My blood sample will be destroyed once these tests have been performed. ☐

Name of Patient _____

Signature of Patient _____ Date _____

Name of Researcher _____

Signature of Researcher_____ Date _____

Original form to be held in University Office, copies to Patient and Hospital Notes

7.8 Sepsis study: Consultee/Relative's Information Sheet

CONSULTEE / RELATIVE'S INFORMATION SHEET v.4 (25 January 2010)

Study Title: Role of nociceptin in sepsis.

Principal Investigator:

Dr Alcira Serrano-Gomez
Specialist Registrar & Honorary Lecturer
University Division of Anaesthesia, Critical Care & Pain Management
Department of Cardiovascular Sciences
3rd Floor, Victoria Building
Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 258 5291 e-mail: asg21@le.ac.uk

Your relative is being invited to take part in a research study. You have been approached as the next of kin as your relative is incapable of making the decision due to illness or sedation. Before you decide for him or her it is important to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Participation is entirely voluntary. If you agree on behalf of your relative to take part, we will ask you to sign a consent form. You are free to withdraw your relative from the study at any time, without giving a reason. If you withdraw, any blood samples your relative has donated will be destroyed.

What is the purpose of this study?

Our laboratory has discovered an increase in blood levels of a 'hormone' called nociceptin when the body is invaded by infection. We believe that this hormone may originate from the white cells in the blood. We wish to measure the production of nociceptin and other stress hormones by these white cells in patients with infection. With the information gained from this study we ***may*** in the future be able to modify how this hormone works, and improve the way the body reacts to infection.

Does my relative have to take part?

No. It is up to you to decide whether or not your relative would wish to take part in this research study.

What will happen if my relative takes part?

Involvement in the study will only last as long as your relative is a patient in the hospital. We will take a total of three small blood samples. The first two samples will be taken from a special cannula (or 'drip) in the artery or vein that will be placed anyway as part of your relative's routine care in ICU, whether or not they are involved in the study. The third recovery sample may be taken with a needle from a vein, if the special cannulas have been removed. The total amount of blood taken will be 75 ml, or approximately half of a tea cup-full.

What are the advantages to my relative of taking part?

The information we obtain from the study will not affect your relative directly, but may help us to improve the treatment that future patients receive.

What are the possible disadvantages and risks to my relative?

We do not anticipate any risk to your relative when donating blood samples. There will be no impact on life insurance or private medical insurance.

What will my relative have to do after the study?

Once the blood samples have been taken, you will have no further involvement in the study. Your recovery after your stay in ICU will continue as it would normally.

Will my relative receive payment for the blood they donate for this research study?

No payments will be made for the samples. The samples are a gift. Neither you nor your relatives will benefit financially from any inventions that result from the use of the blood samples.

What will happen to the results of the study?

Full analysis of the blood samples will take approximately 12 months. The blood samples will be frozen & stored in locked laboratory facilities in the Department of Anaesthesia, Critical Care & Pain Management, Leicester Royal Infirmary. Once the tests on your relative's blood have been performed, any remaining blood samples will be destroyed. It is our intention to publish the results of the study in medical journals and at medical meetings. This may however take a number of years.

Is the study confidential?

All information collected about your relative for this study will be kept strictly confidential, and is locked away in offices & laboratories of the Department of Anaesthesia, Critical Care & Pain Management, Leicester Royal Infirmary. Any information from the study that is reported will **not** use your relative's name or address or any other information that would allow them to be recognised.

What if my relative is harmed by the study?

We do not anticipate your relative being harmed by this study. However, you should know that if they are harmed by taking part in this study, there are no special compensation arrangements. If they are harmed due to someone's negligence then you may have grounds for a legal action, but you may have to pay for it yourself. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you. In the University Hospitals Leicester NHS Trust there is a free & confidential service called the Patient Advice & Liaison Service (PALS). Their contact details are as follows:

PALS Office
Glenfield Hospital
Groby Road
Leicester
LE3 9QP
Tel: 0116 258 3100
e-mail: pals@uhl-tr.nhs.uk

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights, wellbeing and dignity. This

study has been reviewed and given a favourable opinion by this Research Ethics Committee.

Who is organising and funding the research?

This study is being organised and funded by the University Department of Anaesthesia, Critical Care & Pain Management, based at Leicester Royal Infirmary.

Thank you for taking time to read this information leaflet.

7.9 Sepsis study: Consultee Consent Form

CONSULTEE CONSENT FORM

Study Title: Role of nociceptin in sepsis.

Investigators: Dr A Serrano-Gomez, Dr M Wood, Dr N Ladak, Dr J Thompson, Prof D Lambert

Regarding patient (name):_____

Consultee to initialise boxes

6. I confirm that I have read and understood the information leaflet dated 25 January 2010, version 4, and have had the opportunity to ask questions. ☐

7. I understand my role under the Mental Capacity Act 2005 (section 32), which allows me to decide if my relative would want to take part in this study. In my opinion he/she would not object to taking part. ☐

8. I understand that participation in this study is voluntary and that I am free to withdraw my relative at any time without giving any reason, and without any medical care or legal rights being affected. ☐

9. I understand that sections of my relative's medical records may be looked at by responsible individuals from the research team or from regulatory authorities. I give permission for these individuals to have access to my relative's records. ☐

10. I understand that appropriate personal identifying information will be collected, stored and used by the study office to enable follow-up of my relative's health status. This is on the understanding that any information will be treated securely and confidentiality will be maintained. ☐

Name of Consultee _____ Relationship to patient _____

Signature of Consultee _____ Date _____

Name of Researcher _____

Signature of Researcher_____ Date _____

Original form to be held in University Office, copies to Relative and Hospital Notes

7.10 Sepsis study: Research grant letter of approval

-----Original Message-----
From: Lambert, Dave G. (Prof.) [mailto:dgl3@leicester.ac.uk]
Sent: 11 December 2009 15:17
To: 'Thompson, Jonathan'
Cc: 'liz@aagbi.org'; 'igj@doctors.org.uk'; 'David Bogod'; 'Morguler Cenan'
Subject: Anaesthesia/AAGBI Small Grant Application-Funding letter

11th December 2009

Dear Dr Thompson,

The NIAA grants committee with representatives from Anaesthesia/AAGBI and BJA/RCoA met yesterday to consider your application for an AAGBI Small Project grant. I delighted to be able to inform you that your application was recommended for support in the sum of £13,800.

This e.mail is a formal notification of funding for which we would request an acceptance e.mail with cc to all.

For your information I attach a copy of your peer review.

I have also added to the peer review a copy of the abstract from your application that we will post onto the NIAA (and partner) websites along with funding details. Please have a look at this and let me know if there are any (small) changes that you may wish to make.

In order to claim the funding you (or your finance office) will need to contact Anaesthesia/AAGBI directly and for your information I copy the relevant details below. There may be some additional conditions (e.g., the need for interim/final reports) that the project funder will provide.

All funding queries (and especially finance office claims) should be directed to Anaesthesia/AAGBI finance Dept and NOT NIAA.

Ms L Devine liz@aagbi.org
With copy to Finance officer
Ian Johnston igj@doctors.org.uk

AAGBI full contact details are:

21 Portland Place
London W1B 1PY
Telephone: +44 (0) 20 7631 1650,
Fax: + 44 (0) 207 631 4352,

On behalf of NIAA and its funding partners I would like to congratulate you on the quality of your application and look forward to seeing your results published.

With kind regards

Professor DG Lambert
NIAA Grants Officer.

(cc L Devine, Dr Ian Johnston, Dr D Bogod and Morgan Cenan)

7.11 CPB Study: qPCR ΔCt Results for NOP and ppN/OFQ

NOP									
ID	**t0 ΔCt**	**t3 ΔCt**	**ΔΔCt**	**Fold change**	**% knock down**	**t24 ΔCt**	**ΔΔCt**	**Fold change**	**% knock down**
01	6.9	8	1.025	0.49	50.88	9.3	2.39	0.19	80.86
02	4.5	6.2	1.65	0.32	68.14	6.3	1.79	0.29	71.08
03	5.4	6.6	1.23	0.43	57.37	7.6	2.25	0.21	78.98
04	5.38	8.5	3.124	0.11	88.54	6.4	1.074	0.47	52.51
05	5.6	8.8	3.20	0.11	89.12	8	2.36	0.19	80.52
06	9.2	9.6	0.396	0.76	24.05	9.3	0.06	0.96	3.74
07	6.42	6.15	-0.27	1.20		5.85	-0.57	1.48	
08	5.93	7.47	1.54	0.34	65.66	5.99	0.06	0.96	3.94
09	5.24	6.92	1.68	0.31	68.68	5.66	0.41	0.75	24.89
10	6.67	6.37	-0.31	1.24		6.28	-0.40	1.32	
11	6.084	6.447	0.363	0.78	22.25	6.400	0.316	0.80	19.67
12	4.305	5.789	1.484	0.36	64.25	5.291	0.986	0.50	49.51
13	4.929	6.168	1.239	0.42	57.63	5.143	0.214	0.86	13.79
14	5.00	6.1	1.08	0.47	53.0	5.3	0.2	0.85	15.2
15	4.562	5.234	0.672	0.63	37.24	4.687	0.125	0.92	8.30
16	4.716	5.744	1.028	0.49	50.96	6.609	1.893	0.27	73.08
17	4.924	5.869	0.945	0.52	48.06	5.251	0.327	0.80	20.28
18	5.178	6.448	1.270	0.41	58.53	*5.987*	0.809	0.57	42.92

19	4.874	6.211	1.337	0.40	60.42	5.345	0.471	0.72	27.85
20	4.965	5.809	0.844	0.56	44.29	4.941	-0.024	1.02	
21	5.182	5.775	0.593	0.66	33.70	7.089	1.907	0.27	73.34
22	6.144	7.869	1.725	0.30	69.75	6.782	0.638	0.64	35.74
23	4.863	6.410	1.547	0.34	65.78	6.337	1.474	0.36	64.00
24	5.136	5.274	0.138	0.91	9.12	4.583	-0.553	1.47	
25	5.397	7.130	1.733	0.30	69.92	5.716	0.319	0.80	19.84
26	4.747	5.702	0.955	0.52	48.42	4.988	0.241	0.85	15.38
27	4.718	5.781	1.063	0.48	52.14	5.721	1.003	0.50	50.10
28	4.706	6.664	1.958	0.26	74.26	5.965	1.259	0.42	58.22
29	6.046	7.307	1.261	0.42	58.27	7.697	1.651	0.32	68.16
30	3.965	6.138	2.173	0.22	77.83	5.026	1.061	0.48	52.07
31	2.759	5.570	2.811	0.14	85.75	4.310	1.551	0.34	65.87
32	3.630	5.360	1.730	0.30	69.85	4.799	1.169	0.44	55.53
33	4.411	5.581	1.170	0.44	55.56	3.907	-0.504	1.42	
34	4.693	5.691	0.998	0.50	49.93	5.187	0.494	0.71	28.99
35	2.950	4.274	1.324	0.40	60.06	3.774	0.824	0.56	43.51
36	4.166	6.061	1.895	0.27	73.11	5.034	0.868	0.55	45.21
37	5.226	6.357	1.131	0.46	54.34	3.599	-1.627	3.09	
38	4.604	6.227	1.623	0.32	67.53	4.898	0.294	0.82	18.44
39	4.324	5.465	1.141	0.45	54.66	6.277	1.953	0.26	74.17
40	4.691	5.724	1.033	0.49	51.13	5.426	0.735	0.60	39.92

ppN/OFQ									
ID	**t0 ΔCt**	**t3 ΔCt**	**ΔΔCT**	**Fold change**	**% knock down**	**t24 ΔCt**	**ΔΔCT**	**Fold change**	**% knock down**
01	13.36	13.51	0.16	0.90	10.35	14.75	1.40	0.38	62.00
02	ND	13.08				15.92			
03	12.29	16.86	4.57	0.04	95.79	13.99	1.69	0.31	69.08
04	13.87	15.59	1.72	0.30	69.60	16.16	2.30	0.20	79.63
05	6.91	8.09	1.17	0.44	55.66	8.64	1.72	0.30	69.72
06	13.46	16.913	3.450	0.09	90.85	17.901	4.44	0.05	95.39
07	9.28	13.16	3.88	0.07	93.22	13.87	4.59	0.04	95.84
08	11.11	13.57	2.45	0.18	81.74	13.03	1.92	0.26	73.56
09	16.02	19.30	3.28	0.10	89.69	20.37	4.35	0.05	95.09
10	12.85	18.72	5.88	0.02	98.30	16.88	4.04	0.06	93.90
11	14.110	19.095	4.99	0.03	96.84	17.455	3.35	0.10	90.16
12	12.852	18.142	5.29	0.03	97.44	16.960	4.11	0.06	94.20
13	14.381	21.337	6.96	0.01	99.19	21.835	7.45	0.01	99.43
14	15.30	17.40	2.11	0.23	76.80	15.30	-0.04	1.03	
15	14.052	19.927	5.88	0.02	98.30	20.123	6.07	0.01	98.51
16	15.798	21.396	5.60	0.02	97.94	16.750	0.95	0.52	48.31
17	18.411	20.940	2.53	0.17	82.67	ND			
18	12.458	17.084	4.63	0.04	95.95	14.397	1.94	0.26	73.92
19	13.790	19.631	5.84	0.02	98.26	19.333	5.54	0.02	97.86

20	13.080	17.312	4.23	0.05	94.68	17.342	4.26	0.05	94.79
21	15.775	18.062	2.29	0.20	79.51	17.738	1.96	0.26	74.35
22	12.820	18.382	5.56	0.02	97.88	16.320	3.50	0.09	91.16
23	11.524	18.446	6.92	0.01	99.18	14.280	2.76	0.15	85.20
24	13.255	20.776	7.52	0.01	99.46	19.636	6.38	0.01	98.80
25	12.019	21.754	9.74	0.00	99.88	19.227	7.21	0.01	99.32
26	15.155	21.559	6.40	0.01	98.82	18.053	2.90	0.13	86.58
27	11.954	16.424	4.47	0.05	95.49	16.312	4.36	0.05	95.12
28	15.321	25.687	10.37	0.001	99.92	23.806	8.49	0.003	99.72
29	26.963	32.989	6.03	0.02	98.47	30.013	3.05	0.12	87.93
30	13.785	14.408	0.62	0.65	35.07	20.971	7.19	0.01	99.31
31	17.398	19.522	2.12	0.23	77.06	ND			
32	ND	22.377				18.307			
33	ND	17.941				ND			
34	13.510	17.735	4.23	0.05	94.65	ND			
35	ND	ND				16.405			
36	17.025	ND				16.130	-0.90	1.86	
37	14.320	19.790	5.47	0.02	97.74	16.657	2.34	0.20	80.21
38	ND	17.839				ND			
39	ND	ND				ND			
40	15.240	ND				16.530	1.29	0.41	59.10

7.12 Sepsis Study: qPCR ΔCt Results for NOP and ppN/OFQ

NOP									
ID	**D1 ΔCt**	**D2 ΔCt**	**ΔΔCT**	**Fold change**	**% knock down or increase**	**Recovery ΔCt**	**ΔΔCT**	**Fold change**	**% knock down or increase**
01	5.70	5.83	0.14	0.91	↓9.18				
02	7.34	7.46	0.12	0.92	↓7.85				
03	5.80	6.64	0.84	0.56	↓44.22				
04	7.06	7.14	0.08	0.95	↓5.49				
05	8.74	8.88	0.14	0.91	↓9.44				
06	6.25	6.65	0.40	0.76	↓24.21	5.16	-1.09	2.13	↑113.17
07	5.89	5.26	-0.63	1.55	↑55.19	5.82	-0.07	1.05	↑4.97
08	6.01	5.49	-0.52	1.43	↑43.10	5.51	-0.50	1.42	↑41.62
10	7.23								
11	6.20	5.84	-0.36	1.28	↑28.43				
12	3.42	7.20	3.78	0.07	↓92.74				
13	6.96					6.84	-0.12	1.08	↑8.45
14	6.06	5.46	-0.60	1.52	↑51.78				
15	5.33	5.67	0.33	0.79	↓20.61				
16	5.41								
17	5.72	5.74	0.03	0.98	↓1.85				
18	8.10	7.16	-0.95	1.93	↑92.52				
19	6.02	6.28	0.26	0.84	↓16.43				
20	6.80	5.94	-0.86	1.81	↑81.25				

21	7.18	6.44	-0.75	1.68	↑67.60	6.21	-0.97	1.96	↑96.16
22	6.25	7.10	0.85	0.55	↓44.52	6.90	0.65	0.64	↓36.40
23	6.47	6.13	-0.34	1.26	↑26.31	4.78	-1.69	3.24	↑223.57
24	5.84	7.80	1.95	0.26	↓74.17				
25	ND	ND							
26	6.55	5.70	-0.85	1.80	↑80.02				
27	6.02	5.29	-0.74	1.66	↑66.41	1.41	-4.62	24.58	↑2357.55
28	5.42	5.59	0.17	0.89	↓10.82	5.00	-0.43	1.34	↑34.47
29	3.30	3.23	-0.08	1.05	↑5.33				
30	7.71	7.38	-0.33	1.26	↑25.57	6.42	-1.29	2.44	↑144.02
32	7.20	6.76	-0.44	1.35	↑35.27				
33	6.15	6.35	0.20	0.87	↓12.83	7.51	1.36	0.39	↓60.90
34	6.48	5.83	-0.65	1.57	↑56.77				
35	5.39	5.89	0.50	0.71	↓29.21	4.60	-0.79	1.73	↑73.30
36	7.36	5.40	-1.97	3.91	↑291.45	7.59	0.22	0.86	↓14.33
37	4.64	4.59	-0.05	1.04	↑3.72				
38	6.56	6.89	0.33	0.80	↓20.49	5.42	-1.14	2.21	↑121.04
39	5.22					5.68	0.46	0.73	↓27.25
40	7.28	5.20	-2.07	4.21	↑320.79				
41	5.43	4.77	-0.65	1.57	↑57.21	5.60	0.17	0.89	↓11.11
42	7.06								
43	5.52	2.43	-3.09	8.49	↑749.36	5.06	-0.46	1.37	↑37.40
44	2.74	4.64	1.90	0.27	↓73.26				
45	5.09	4.45	-0.64	1.56	↑55.61	4.34	-0.75	1.68	↑67.83

46	3.26								
47	4.83	4.96	0.13	0.91	↓8.83	4.75	-0.08	1.06	↓5.55
48	5.75	6.63	0.88	0.54	↓45.51	3.94	-1.82	3.52	↑252.34
49	7.77	7.28	-0.50	1.41	↑40.96				
50	5.31	4.08	-1.23	2.34	↑134.20	4.93	-0.38	1.30	↑29.90
51	4.62	4.12	-0.50	1.42	↑41.71	4.36	-0.26	1.20	↑19.95

ppN/OFQ									
ID	**Day 1 ΔCt**	**Day 2 ΔCt**	**ΔΔCT**	**Fold change**	**% knock down or increase**	**Recovery ΔCt**	**ΔΔCT**	**Fold change**	**% knock down or increase**
01	15.86	17.70	1.84	0.28	↓72.11				
02	17.95	15.07	-2.88	7.36	↑635.95				
03	12.78	10.66	-2.11	4.33	↑332.87				
04	18.82	15.53	-3.29	9.79	↑878.64				
05	18.53	16.30	-2.23	4.68	↑368.46				
06	14.66	13.61	-1.06	2.08	↑108.35	12.83	-1.84	3.57	↑257.02
07	17.59	17.34	-0.25	1.19	↑19.09	15.74	-1.85	3.60	↑260.00
08	18.50	17.63	-0.87	1.83	↑82.64	18.67	0.17	0.89	↓10.87
10	ND								
11	16.33	13.79	-2.54	5.81	↑481.19				
12	ND								
13	12.28					13.47	1.20	0.44	↓56.35
14	14.47	15.09	0.62	0.65	↓35.02				
15	16.24	16.17	-0.07	1.05	↑4.83				
16	14.75								

17	13.07	10.91	-2.16	4.47	↑346.91				
18	15.80	16.66	0.86	0.55	↓44.98				
19	16.50	13.40	-3.10	8.59	↑758.61				
20	13.75	13.01	-0.74	1.67	↑66.90				
21	16.87	17.23	0.36	0.78	↓21.81	14.56	-2.31	4.97	↑397.26
22	14.17	16.55	2.37	0.19	↓80.67	12.51	-1.66	3.17	↑216.67
23	16.22	15.82	-0.41	1.33	↑32.50	16.56	0.33	0.79	↓20.68
24	11.14	14.83	3.69	0.08	↓92.22				
25	ND	ND							
26	17.19	17.41	0.21	0.86	↓13.79				
27	17.05	16.01	-1.04	2.05	↑105.45	14.30	-2.75	6.73	↑573.44
28	16.95	15.77	-1.18	2.27	↑126.74	16.18	-0.77	1.70	↑69.94
29	11.53	14.33	2.80	0.14	↓85.67				
30	17.28	15.33	-1.95	3.86	↑286.00	12.55	-4.73	26.56	↑2556.42
32	19.80	20.59	0.80	0.58	↓42.49				
33	14.29	13.51	-0.77	1.71	↑70.99	11.55	-2.73	6.65	↑565.14
34	16.54	15.96	-0.57	1.49	↑48.94				
35	16.02	21.96	5.93	0.02	↓98.36	17.94	1.91	0.27	↓73.43
36	17.66	16.23	-1.43	2.69	↑168.66	14.52	-3.13	8.77	↑777.28
37	13.25	12.78	-0.48	1.39	↑39.16				
38	19.48	ND				13.73	-5.75	53.84	↑5283.57
39	ND					8.97			
40	17.19								
41	5.43	4.77	-0.65	1.57	↑57.21	5.60	0.17	0.89	↓11.11

42	20.69								
43	ND	15.00				13.36	-1.64	3.12	↑212.03
44	17.18	17.29	0.11	0.93	↓7.09				
45	16.63	ND				14.11	-2.52	5.75	↑475.07
46	17.61								
47	18.16	16.96	-1.20	2.30	↑129.87	12.04	-6.12	69.57	↑6856.84
48	15.45	14.09	-1.36	2.56	↑156.45	10.21	-5.24	37.90	↑3690.48
49	11.71	14.13	2.42	0.19	↓81.27				
50	14.05	14.22	0.17	0.89	↓11.11	10.73	-3.33	10.04	↑904.14
51	19.68	16.65	-3.03	8.17	↑716.53	11.30	-8.39	334.94	↑33394.26

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